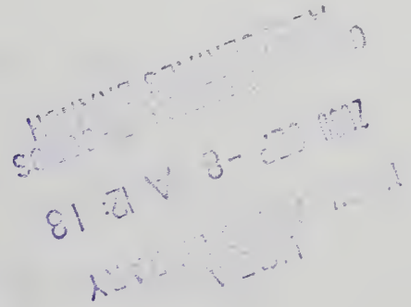
AFLATOXIN ELIMINATION WORKSHOP

Little Rock, Arkansas
October 25-26, 1993



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Aflatoxin is recognized as a serious food safety hazard by most countries of the world. Producing food free of aflatoxin today requires a truly national effort and, particularly, the cooperation of both government and industry. The Agricultural Research Service (ARS) and the commodity groups representing peanuts, corn, cottonseed, and tree nuts recognize the importance of a strong national research effort to eliminate aflatoxin as a food safety threat.

This Aflatoxin Elimination Workshop, held in Little Rock, Arkansas, is the sixth such yearly meeting held to review the ARS supported aflatoxin research and provide a forum for interested scientists to come together to discuss common problems and their potential solutions among themselves and with members of the industry. Thus, although many of these scientists are performing very fundamental studies researchers and representatives of Industries affected by aflatoxin who attend the workshop gain a very clear idea of where their research is leading and the impact it will have on society. Also, this workshop provides the opportunity for gains in cost effectiveness of research by the recognition of common approaches and by sharing relevant information across commodities. This workshop has come to be recognized as the premier national meeting for advances leading to methods to eliminate aflatoxin.

Yearly advances are being made in understanding many aspects of aflatoxin contamination of food crops, including the plant/fungal/environmental relationships, molecular biology, and identification and utilization of resistant germplasm. I am particularly pleased that the molecular biology approaches being used have allowed the cooperating laboratories to isolate several enzymes and genes in the biosynthetic pathway and to study their regulation, thereby providing insight into how the fungus produces aflatoxin. Sufficient information is now available to provide the basis for a strategy to disrupt several large segments of the biosynthetic pathway. Evidence is also accumulating that specific compounds in crop plants provide an important mechanism to inhibit aflatoxin biosynthesis. This could lead to the development of a gene specific assay to screen plant genotypes based on the ability of plant extracts to inhibit not only growth of toxin producing fungi, but also aflatoxin pathway genes.

Most of the research is performed by the ARS, however an important addition to this core effort is provided through a competitive award program provided by Congressional appropriations. This program is a unique effort of the ARS and representatives of the peanut, corn, cotton, and tree nut industries. By extending the opportunity for the best university scientists to join the highly focused multithrust program, the rate of progress toward the elimination of aflatoxin is enhanced.

On the following pages are the abstracts of work presented at the 1993 Workshop.

Jane F. Robens

JANE F. ROBENS
Agricultural Research Service
Workshop Program Coordinator

December 9, 1993

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AFLATOXIN ELIMINATION WORKSHOPS

New Orleans, LA	1988
Peoria, IL	1989
St. Louis, MO	1990
Atlanta, GA	1991
Fresno, CA	1992
Little Rock, AR	1993
St. Louis, MO	1994

COOPERATING COMMODITY GROUPS

PEANUTS:	National Peanut Council
CORN:	National Corn Growers Association American Corn Millers Federation Corn Refiners Association
COTTONSEED:	National Cottonseed Products Association National Cotton Council
Tree Nuts:	Prune, Raisin & Walnut Marketing Board Almond Board of California California Pistachio Commission DFA of California

PLATFORM PRESENTATIONS

Crop Management and Handling, Insect Control and Plant Fungal Relationships

Aflatoxin Control in Pistachio, Fig and Walnut: Ecology, Agronomic Practices and Characterization of Contaminated Nuts/Fruits.

Mark A. Doster and Themis J. Michailides. Department of Plant Pathology, University of California, Davis, Kearney Agricultural Center, Parlier 93648.

Aspergillus molds frequently infested, infected, and sporulated on pistachio (*Pistacia vera*) litter such as fallen fruits and male inflorescences throughout summer in commercial pistachio orchards in California. *A. niger* was isolated much more frequently from pistachio litter than any other species but *A. flavus* and *A. parasiticus* also frequently developed in pistachio litter. Two distinct strains of *A. flavus* were isolated: strain L (few large sclerotia) and strain S (abundant small sclerotia). Strain L occurred substantially more frequently than strain S. Although all isolates of strain S and *A. parasiticus* were aflatoxin producers, only 43% of the isolates of strain L produced aflatoxins. *A. flavus* and *A. parasiticus* were found in litter in all nine orchards surveyed. The development of *Aspergillus* molds in pistachio litter could increase the amount of *Aspergillus* inoculum in the orchard, resulting in increased numbers of moldy and mycotoxin-contaminated pistachio nuts.

Early splits (ES) are atypical pistachio nuts that have a split hull, exposing the kernel to invasion by molds and insects. Normal nuts have intact hulls. A total of 14 different *Aspergillus* species were isolated from the kernels of pistachio nuts, mainly ES, from 11 commercial orchards in California. Although *A. niger* was the most commonly isolated species, *A. flavus* and *A. parasiticus* were also found in ES from most orchards. The incidence of ES nuts varied from 0.8 to 4.6% in commercial orchards using various cultural practices. Only irrigation practices affected the percentage of ES formed. Percentage of ES showed trends towards slightly increased levels with increased irrigation (results from 1992 and 1993). In addition, in two commercial orchards, skipping one irrigation during the period of shell formation (around mid May) resulted in higher (almost twice as high) incidence of ES than irrigating normally (Table 1). Management of irrigation schedule can reduce the incidence of ES, resulting in lower incidence of mycotoxin-contaminated nuts because almost all aflatoxin contamination was associated with ES.

The time of ES formation was determined by marking pistachio nuts with yarn as they developed in two commercial orchards. The majority of the ES developed in the second and third week in August, although some developed before or after that period. ES nuts that developed before and during the second and third week in August had significantly smaller shell dimensions and significantly more staining than those that developed after the third week in August. In addition, the earlier the ES developed the smaller the fruit and hull weights (both fresh and dry), the lower the moisture of their hulls and kernels, and the higher the incidence of hull shriveling. Most importantly, ES that developed before the fourth week in August had higher incidence of *Aspergillus* molds and infestation by the navel orangeworm (NOW). These NOW-infested ES with rough, shriveled hulls contained 99.9% of the aflatoxin detected. Therefore, reducing the incidence of ES development with proper irrigation or separating them based on the special size, staining, and moisture content characteristics can reduce aflatoxin contamination in pistachio.

Aspergillus flavus and *A. parasiticus* were isolated from soils of all eight Calimyrna fig orchards surveyed. Studies with walnuts have been initiated.

TABLE 1. Effect of skipping one flood irrigation during the shell formation period on incidence of early split (ES) pistachio nuts.

Irrigation treatment	Date counted	Early splits (%)	Date counted	Early splits (%)
	Delano orchard:		**Stockton orchard:**	
Normal	27 Aug	4.3	1 Sep	3.0
Skip one		9.2*		5.5*
Normal	6 Sep	5.8	13 Sep	4.2
Skip one		10.7*		6.5*
	$LSD_{0.05}$	1.4		1.3

* Significant differences.

Relevant Publications (Last 2 Years)

1. Doster, M. A., and T. J. Michailides. 1993. Characteristics of pistachio nuts with *Aspergillus* molds and aflatoxins. Phytopathology 83: (submitted).

2. Doster, M. A., and T. J. Michailides. 1993. Development of *Aspergillus* molds in litter from pistachio trees. Plant Disease 77:(submitted).

3. Doster, M. A., T. J. Michailides, and D. A. Goldhamer. 1993. Influence of cultural practices on occurrence of early split pistachio nuts. California Pistachio Annu. Report. Crop Year 1992-1993. California Pistachio Commission, Fresno. pp. 82-84.

4. Doster, M. A., and T. J. Michailides. 1993. Characteristics of pistachio nuts with *Aspergillus* molds. California Pistachio Annu. Report. Crop Year 1992-1993. California Pistachio Commission, Fresno. pp. 64-68.

5. Doster, M. A., and T. J. Michailides. 1992. Ecology of *Aspergillus* molds in pistachio orchards. California Pistachio Annu. Report. Crop Year 1991-1992. California Pistachio Commission, Fresno. pp. 101-104.

6. Michailides, T. J., and M. A. Doster. 1993. Possible involvement of hemipterans in contamination of pistachio nuts with *Aspergillus flavus*. California Pistachio Annu. Report. Crop Year 1992-1993. California Pistachio Commission, Fresno. pp. 98-99.

7. Michailides, T. J., and M. A. Doster. 1993. Aflatoxin and mycotoxins in figs. Annu. Fig Research Report. California Fig Institute, Fresno. 5 pp.

EXPERIMENTAL DETERMINATION OF AFLATOXIN DISTRIBUTION OF PROCESSED PISTACHIOS

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The aflatoxin concentration in 20 samples of 10 nuts each has been measured in sets of lots representing commercial process-sorted pistachios. The protocol used 60% MeOH extraction, immuno-affinity column [VICAM] column purification and solvent exchange, pre-column derivatization with TFA and HPLC/fluorescence detection. Sensitivity was 0.1 ppb, accuracy was 0.25 ppb plus 5%. The results are shown in the table.

Aflatoxin B_1, ppb in 20 samples of 10 nuts each

Process	Sinkers	Floaters
Meats	none	0.1, 0.2 (14), 0.3 (2)
Needle Pick	none	none
Air Light	none	0.1, 0.02 (6), 0.4, 0.5
Eye Reject	none	0.1 (6), 0.2
HPO Insects	0.7	n.a.
HPO Shell Stock	none	0.1, 0.3
HPO Dye Stock	none	0.1 (6), 0.5
Small (>30/on.)	0.1, 0.2	0.2 (2)
Large (22/on.)	none	0.1 (3)
Extra Large (19.5/on.)	0.1 (0.6)	0.1 (3)

Lots were all from a single processor and 1992 crop. Floaters and Sinkers refer to water floatation carried out after hulling and before drying for storage. Process streams are given in sort order upon removal from storage. HPO refers to hand sorting. Numbers in brackets indicate multiple samples. The results show a strong bias towards floaters and insect infestation and a secondary bias towards low quality product. Work in progress suggests that these results are maintained with larger samples, which also show higher aflatoxin levels.

We postulate that floatation is able to select early split nuts which are correlated with aflatoxin content. We base this on the following. Sommer [Phytopath. 76:692 (1986)] showed that hanging nuts contain aflatoxin only if the hull surrounding the nut splits prematurely. Work by Slaughter, Studer and Pearson [these proceedings] indicates that early split causes hull adhesion. Processing practice suggests that hull adhesion causes floatation. Our results are consistent with that scenario. Since floaters comprise only about 10% of total product and aflatoxin is concentrated in 1/3 of this, while HPO Insects amount to ~1% of Sinkers it should be possible to eliminate most of the field source aflatoxin by removing 4% of final product and less of final value. These results are tentative and need to be verified with larger samples and additional processors.

Effects of Meteorological Factors on Pre-Harvest Formation of Aflatoxin in Cottonseed

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ABSTRACT

This study focused on determining whether meteorological factors play a role (1) in *A. flavus* contamination in cotton growing areas and (2) aflatoxin formation in cottonseed prior to harvest for various locations in Arizona.

Selected cotton fields located in Yuma, Casa Grande, Buckeye and Mohave were monitored for *A. flavus* contamination and various meteorological factors (day and night-time temperature, relative humidity, wind speed, and rainfall) during the cotton growing season. As the cotton plant matured, aflatoxin formation was determined in cottonseed harvested from the fields, trailers or modules, and cottonseed after the ginning operation.

The monitoring of meteorological factors for the sample sites revealed similar patterns for temperature and relative humidity. Values observed would favor the growth of *A. flavus* and production of aflatoxin during the cotton growing season. Very low rainfall was recorded for all sites, especially in Mohave and Yuma from May onward. Significant *A. flavus* contamination was found at all monitoring sites with higher levels of mold contamination on the lower portion of the plant. Aflatoxin contamination in cottonseed was observed during the growing season (1-98 ug/kg range), although the overall averages observed were considered low in comparison to other years. Two "pockets" of aflatoxin contamination were found in Buckeye at levels of 98 and 95 ug/kg; however, the average observed for Buckeye after harvest was less than 3 ug aflatoxin/kg.

Aflatoxin Concentration in Peanuts as Affected by Calcium and Boron

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Calcium and boron deficiencies can affect the biological integrity of peanuts. Aspergillus flavus invasion of seed and subsequent aflatoxin contamination, is more likely to occur when the seed integrity is compromised. The objective of these experiments was to determine the effect of calcium and boron on the incidence of A. flavus and aflatoxin. Six on-farm experiments were initiated in 1991. A split plot design was used with cultivars ('Florunner, Sunrunner, and Gk 7) as the whole plots and gypsum application as the subplots. Only two experiments had measurable aflatoxin concentrations and none of the experiments had increased yield due to gypsum. In 1992, three calcium and two boron experiments were initiated. Only one calcium experiment had increased yield and sound-mature kernels (smk) due to gypsum. All other experiments from 1991 and 1992 did not have increased yield due to treatments. Incidence of A. flavus increased with decreasing seed calcium and soil calcium. Aflatoxin was erratic across all experiments. Boron appeared not to affect incidence of A. flavus. Also the incidence of A. flavus decreased as soil pH increased. Further research should be directed to separating the effects of soil calcium and soil pH on incidence of A. flavus in peanut.

Examining the Biosphere for Factors which Influence Aflatoxin Contamination in Preharvest Corn

N. W. Widstrom, D. M. Wilson, J. A. Lansden, J. I. Davidson, and M. C. Lamb

The corn farmer needs additional management information to assist him in keeping his crop free of contamination by aflatoxin. Our objective is to monitor numerous environmental factors and management components to determine how they influence field aflatoxin contamination. Four widely separated, south Georgia irrigated cornfields were monitored. Two of these locations were also used to provide information for non-irrigated fields. The following data were collected:

1. Ambient temperature
2. Canopy temperature
3. Canopy relative humidity
4. Leaf wetness
5. Ear temperature
6. Soil moisture (8" and 18" depth)
7. Rainfall + irrigation
8. Complete soil test at denting stage
9. Ear samples weekly beginning at three weeks post-silking
   a. aflatoxin analyses
   b. kernel infection percentage.

Data for traits 1 through 7 were recorded on an hourly basis from just prior to silking through harvest.

Some pairs of sensors measure variables that are very closely related; therefore, one or more of these sensors may not be needed in our final model for predicting probable contamination of the corn crop. Examples of closely related variables were: canopy temperature and ear temperature; leaf wetness and canopy relative humidity; and canopy temperature and ambient temperature. Measurable levels of aflatoxin occurred in one irrigated and one non-irrigated field. No aflatoxin occurred prior to three weeks before harvest. The variables found to be most closely associated with aflatoxin contamination were leaf wetness, ear temperature, and ambient temperature. Similarly, increased levels of kernel infection by A. flavus occurred at three of four locations by nine weeks after silking. The variables most closely associated with A. flavus infection were canopy relative humidity, rainfall + irrigation, canopy temperature, and ear temperature. The correlation of soil analyses between pairs of locations was highest between the two locations with verifiable levels of aflatoxin just prior to harvest.

Time of Kernel Infection After Inoculation of Maize Ears with A White and A Green Isolate of *Aspergillus flavus*

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Knowledge of the kernel infection process by *Aspergillus flavus* in corn is important in order to efficiently inoculate and identify resistant genotypes in the field. Earlier studies, using white and green isolates of the pathogen, showed that kernel infection probably occurs through the pericarp. Similar studies showed that needle inoculation of ears could be carried out over a period of several days with little effect on infection levels. However, these studies did not indicate how long it took for kernels to become infected after the fungus entered the ear. In order to determine how long it took for kernel infection to occur, ears of Pioneer brand 3369A in replicated plots were inoculated at 6 days after mid-silk with a white isolate (NRRL 20521) or a green isolate (NRRL 3357) of *Aspergillus flavus* in 1992 and 1993. Ten ears from each of 12 plots were harvested at 1, 2, 3, 4, 5, 10, and 20 days after inoculation, dipped momentarily in 70% ethanol, immersed for 3 min in 1.5% NaOCl, and rinsed in sterile distilled H_2O. The ears from each plot were placed in individual sterile moist chambers and incubated at 28 C for 7 days. Infection percentage was determined for each harvest day by counting the number of kernels on each ear and determining the number of kernels that showed external growth of the fungus. We found that both isolates of the fungus could infect kernels 24 hr after inoculation. There was an increase in percent infection from day 1 to day 10 after inoculation.

Sample Size Needed to Determine Kernel Infection Levels by *Aspergillus flavus* in Maize

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There is always the question of how many kernels are needed in a sample to determine the percentage of infection by a pathogen to obtain the level of differences among treatments that we desire. To evaluate for differences among treatment and/or genotypic means, we basically grow six replications and evaluate 390 kernels per plot. The kernels are surface sterilized and then plated on Czapek solution agar amended with 7.5% NaCl in Pitre plates with 13 kernels per plate. Plates are incubated for seven days at 28 C and the percentage of infected kernels is calculated. Thirteen kernels in each of 30 plates provides a convenient way to study different sample sizes. We took groups of five plates or multiples of five plates to get six samples of 65 kernels, three samples of 130 kernels, and two samples of 195 kernels.

Relative variability within a test can be measured by comparing error mean squares, F-values, r^2-values, C.V.'s, etc. Simulated data indicated that the greatest differences between sample sizes occurred for error mean squares and F-values for entries. With the simulated data, error mean squares were some six times larger with sample size of 65 kernels compared to sample size of 390 kernels. Differences between F-values for these two sample sizes were of the same magnitude. The average error mean squares for all experiments of actual data were 4.2 times greater than for simulated data with 390 kernels sample size and the differences in F-values were much larger. This should not be surprising when we are evaluating single crosses because the female portion of the kernels, except for the pericarp, are a F_2 population and the male portion of the kernels are a random sample of pollen present in the field. Error mean squares were 1.8 times greater with 65 kernels than with 390 kernels with data from actual experiments. F-values were 1.5 times larger with 390 kernels than with 65 kernels.

Error mean squares for sample sizes of 65 kernels were quite large and F-values were low indicating that this sample size is too small to detect differences among treatment means at a desirable level. Differences in mean squares and F-values between sample sizes of 195 and 390 kernels were relatively small. Perhaps sample sizes of around 200 kernels will provide a sufficient level of precision with the minimum amount of input.

Of course, we want better precision in determining treatment means, but we also want repeatability in ranking of treatment means. Spearman rank correlation coefficients were calculated within each sample size. The average "r" values over all experiments were 0.779, 0.859 and 0.912 for sample sizes of 65, 130, and 195 kernels, respectively. Thus, sample sizes of 65 kernels were definitely poorer than sample sizes of 195 kernels.

Managing Aflatoxin Contamination of Cottonseed and Corn Kernels in a High Stress Environment

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In the second year of this study, we have continued our work with corn and initiated the study on aflatoxin contamination of cottonseed. Here we report on the second years's efforts with corn and first-year results of our work in cotton.

Corn. The previous year's results indicated that: 1) regular irrigation did not prevent aflatoxin contamination, 2) insect damage, by itself, was not considered to be a direct cause of aflatoxin contamination in the ear, 3) low humidity and high temperatures were closely linked with the onset of contamination. Therefore, the same 6 varieties used in the previous year were established in: 1) a fall planting and 2) early/late spring planting in an attempt to impose different temperatures and humidity during kernel maturation. Varieties planted and inoculated in the fall accumulated only 5 to 20% of the aflatoxin levels found in the same varieties planted in the spring. Following wound inoculation with a local isolate of *A. flavus*, the early-planted varieties accumulated aflatoxin concentrations 2 to 4 times higher than the planting made 30 days later. However, aflatoxin levels in the non-inoculated controls were highest in the later planting. The environmental advantages conferred by early planting appeared to be circumvented with wound inoculation. After two years of examining fall and spring plantings, decreased susceptibility to aflatoxin contamination is consistently associated with the tight husk character. Decreased susceptibility seems to result from a direct effect on kernel development rather than the ability of tight husks to exclude insects or fungal contamination. Creating wound sites through the husk did not compromise the ability to protect against aflatoxin contamination. Susceptibility to contamination is associated with looser husks and the tendency to develop silk-cut or other kernel fractures during periods of low rainfall, high rates of evaporation, and excessive heat.

Cotton. Data obtained from Valley Co-op Oil Mill indicates that approximately 38% and 16% of the incoming cottonseed exceeded a self-imposed action threshold of 15 ppb aflatoxin in 1992 and 1993, respectively. Over 6000 truckloads representing approximately 140,000 tons of seed are sampled annually and tested for aflatoxin. The mill services cotton gins located throughout the Coastal Bend area of Texas beginning south of Houston and extending into the Lower Rio Grande Valley with additional cottonseed coming from Uvalde county (Winter Garden area) located just west of San Antonio. However, aflatoxin contamination is not uniform across all gin locations or processing dates. The highest levels of aflatoxin contamination occur in a 150 mile band of the middle and upper Coastal Bend area. The percentage of contaminated seed gradually increased during the later portion of the season. In 1993, the percentage of contaminated seed above 15 ppb increased from near 10% to over 50% during the last

30 days of processing. There is published information suggesting that older bolls on the lower part of the plant may be a major source of the aflatoxin contamination in cottonseed. The early bolls are subjected to prolonged exposure and become more vulnerable to environmental and biological stresses. Consequently, our initial efforts were directed at defining the contribution of these older bolls to aflatoxin contamination in cottonseed and the effect of their removal on the severity of contamination. In a replicated study over 2 years with 'DPL 50', bolls were harvested from the upper and lower half of cotton plants left untreated or treated with Ethephon to eliminate the earliest bolls on the lower portion of the plant. In the upper half of the plants from the 1992 experiments, aflatoxin levels were 0 and 30 ppb for the control and Ethephon treatment, respectively. Seed from bolls in the lower half measured 27 and 50 ppb for the control and Ethephon treatment, respectively. The aflatoxin levels in the 1993 experiments were much lower but reflected the same tendencies. Our results suggest that seed from the older bolls on the plant may be more vulnerable to aflatoxin contamination. In an additional study for 1993, the natural levels of aflatoxin and *A. flavus* were measured in the lower bolls from untreated plants for 6 weeks at weekly intervals prior to harvest. The number of *A. flavus* propagules on the boll was highest, 275 propagules/gm, during the first week after boll opening and declined to less than 25 propagules/gm within the next 5 weeks. Aflatoxin concentrations in the seeds were generally less than 5 ppb and insufficient to make any quantitative comparisons with *A. flavus* levels on the bolls. The mild weather conditions may have decreased the incidence of aflatoxin contamination in this year's crop with the exception of the late harvests. Survey data from the mill suggests that bolls harvested later in the season may have developed a higher level of aflatoxin contamination.

Summary: Panel Discussion on "To What Degree Can Optimum Crop Production and Handling Practices Eliminate Aflatoxin?"

The panel consisted of: P. Cotty, P. Dowd, J. Dunlap, T. Michailides, D. Park, T. Schatzki, and N. Widstrom. The discussion was chaired by G. Obenauf and the summary was prepared by P. Cotty.

Research findings presented in this portion of the workshop demonstrated an increased knowledge of the ecology of aflatoxin producing fungi and an improved understanding of the processes which lead to aflatoxin contamination of various crops. This combined knowledge base is leading to insights concerning how crop management techniques might be optimized in order to minimize aflatoxin contamination.

Dr. Michailides presented evidence for the association of most aflatoxin contamination of pistachios with early splitting in the field. Association of contamination with specific nut types was corroborated by Dr. Schatzki. Dr. Michailides further demonstrated that early splitting of pistachios could be greatly reduced in the California growing areas through proper management of irrigation practices. The panel was questioned as to whether growers would be willing to adopt such management procedures in order to limit contamination. After discussion among the panel and other attendees, it was agreed that control through careful management is often highly cost effective and that this is a type of pest control that growers are accustomed to implementing. Changing the timing of irrigations may have little or no cost and thus be readily accepted by Pistachio growers.

It was also mentioned that recommendations for minimizing aflatoxin contamination of cottonseed also exist. These require early harvest, control of insect damage during early boll development, and proper moduling and handling of the crop. In some areas and years these recommendations are followed. However, weather and/or resource constraints can often lead growers to abandon recommended control procedures. Grower incentives to manage aflatoxin contamination during crop development may have to be increased for certain crops and/or localities.

Further advances in the management of contamination through cultural methods are being sought by panel members for cotton (Park and Dunlap), corn (Scott, Zummo, Dunlap, and Widstrom), peanuts (Cole and Demski), and tree crops (Schatzki and Michailides). These include procedures to optimize the timing of crop planting and harvest, models to predict the impact of multiple interacting variables on contamination, efforts to identify the most contaminated components of the crops, and integrated approaches (Dowd and Vega).

Comments from the floor questioned if cultural management could really eliminate contamination or whether it would only reduce contamination levels. It was agreed that cultural management probably would only reduce contamination levels, but that such reductions would be valuable. Many on the panel felt that to achieve consistent,

acceptable control of contamination, an integration of several methods including genetic control may be necessary. The discussion also included the concept of a zero aflatoxin level. Zero levels are defined by the detection threshold of the method employed. The heterogeneity of aflatoxin contamination dictates that precise sampling be undertaken in order to detect aflatoxins at the level dictated by regulations. Therefore, research goals should seek toxin below a certain practical level, not at zero. At the same time, it was recognized that the level of tolerable aflatoxin differs by crop with feed crops tolerant of higher levels than food crops.

PLATFORM PRESENTATIONS

Microbial Ecology

Management of aflatoxin contamination of cottonseed in Arizona

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The main objective of the study is to develop optimal procedures to reduce aflatoxin contamination of cottonseed in Arizona. Among several hundred bacterial isolates, recovered from cotton plants and soils throughout Arizona, one isolate (D1) seems to be promising as a biocontrol agent against Aspergillus flavus. The isolate which was found to be a strain of Pseudomonas cepacia produces antibiotics against A. flavus; prevents sporulation of the fungus in culture, on cottonseed, and on organic debris; inhibits germination of sclerotia of the fungus; and reduces the level of boll rot in the greenhouse and in the field.

Results of field trials to test the efficacy of D1 to reduce aflatoxin contamination of cottonseed in the 1992 season were inconclusive because of the low incidence of natural A. flavus infection in the field and an unusually heavy white fly infestation. However, results of field trials in 1993 were encouraging. The percentage of A. flavus-infected cottonseed from plants in a field plot, not treated with D1, was more than 11 fold greater than that of those from plants spray inoculated with D1. The difference was statistically significant ($p < 0.05$).

Isolate D1 survived on leaf, boll, and soil surfaces in the field up to two weeks after its introduction into these sites. Populations of both D1 and A. flavus were significantly greater on boll surfaces than on leaf surfaces in field-grown plants. Application of D1 to field-grown plants using an aqueous spray suspension containing a commercially available sticker at 1 % resulted in recovery of a significantly higher number of the bacterium from leaf and boll surfaces after a heavy rainfall, compared to plants spray inoculated with D1 using an aqueous suspension without sticker.

Concomitant Use of *Aspergillus flavus* and *A. parasiticus* for Reducing Preharvest Aflatoxin Contamination of Peanuts

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Studies conducted at the National Peanut Research Laboratory between 1987 and 1991 demonstrated great potential for biological control of preharvest aflatoxin contamination of peanuts through the application of non-toxigenic strains of *Aspergillus parasiticus* to soil. Data gathered during these years indicated that introduction of a large population of the non-toxigenic strains prevented the normal buildup of aflatoxigenic strains of *A. flavus* and *A. parasiticus* that normally occurs late in the peanut growing season during years of severe drought stress. The result of this prevented buildup was reduced levels of aflatoxin in peanuts.

Studies were expanded in 1992 to test other strains of *A. flavus* and *A. parasiticus* as potential biocontrol agents against preharvest aflatoxin contamination of peanuts. These included a strain of *A. flavus* that produced neither aflatoxin nor cyclopiazonic acid, another common mycotoxin of *A. flavus*; the original strain of *A. parasiticus* that had previously been shown to be effective; a combined application of the *A. flavus* and *A. parasiticus strains*; and two non-toxigenic color mutants of *A. parasiticus*. Results of these studies showed dramatic reductions in aflatoxin contamination of peanuts in all treatments, but particularly for the treatment that included both the non-toxigenic strains of *A. flavus* and *A. parasiticus*. In that treatment aflatoxin contamination of edible category peanuts was only 0.8 ppb compared with 75.1 ppb in peanuts grown in untreated soil.

For studies conducted in 1993, a UV color mutant of the *A. flavus* strain used in 1992 was developed. Two environmental control plots at the National Peanut Research Laboratory were used to test the efficacy of this color mutant used in combination with one of the *A. parasiticus* color mutants found to effective in 1992. Three environmental control plots were used to further test the *A. flavus*/*A. parasiticus* combination used in 1992. The mean aflatoxin content of edible peanuts from all treatments was 4.0 ppb compared with a mean from untreated soils of 93.8 ppb. The color mutants appeared to as effective as the originally tested strains as the mean aflatoxin from the color mutant treatments was 5.3 ppb compared with a mean of 3.2 ppb in peanuts from the treatments with the original *A. flavus*/*A. parasiticus* combination. Results of soil population analyses revealed that application of the *A. flavus*/*A. parasiticus* color mutants had a significant effect on the buildup of wild strains of *A. flavus*/*A. parasiticus* in soil. At harvest, soil populations of wild strains of *A. flavus*/*A. parasiticus* averaged 5490 colony forming units per gram of soil (CFU/g) in the untreated plots compared with 211 CFU/g in plots treated with the *A. flavus*/*A. parasiticus* color mutants.

Results of these studies have shown that the most effective biological control against preharvest aflatoxin contamination of peanuts to date was achieved with a combination of non-toxigenic strains of *A. flavus* and *A. parasiticus*. This effectiveness appears to result from an exclusion of toxigenic strains of *A. flavus*/*A. parasiticus* in peanut soils that have been inoculated with the biocontrol strains.

Aflatoxin Elimination Workshop, 1993
Little Rock, Arkansas

Geocarposphere Bacteria as Biocontrol Agents for the Minimization of Aflatoxigenic Fungal Invasion of Peanuts. K.L. Bowen and C.J. Mickler, Dept. Plant Pathology, Auburn University, AL 36830-5409

The geocarposphere, or area immediately surrounding peanut pods, is a unique ecological habitat. One hundred fifty-nine bacterial strains were isolated from the geocarposphere during several sampling periods in 1990. These strains were tested for inhibition against *Aspergillus flavus*-type fungi using *in vivo* assays. Nineteen strains were selected from the collection based on their ability to inhibit fungal growth and slow conidiation of *A. flavus*-type fungi. One of these strains significantly ($P < 0.10$) increased root growth during germination of peanut seed. Three strains were then used to determine optimum time of application for a field study. Comparisons were done in a greenhouse trial between seed bacterization (soaking seed in each of the bacterial strains prior to planting) and mid-peg application (soil drench of bacterial suspension). Seed bacterization resulted in less seed invasion by aflatoxigenic fungi at maturity.

In the field trial, potential biocontrol strains were applied by both methods (seed bacterization at-plant and soil drench at mid-peg) within each plot. Developing pegs and pods were sampled every two weeks and assayed for aflatoxigenic fungal invasion starting 28 July (at mid-peg, just prior to soil drench treatment). Performance of several bacterial strains toward inhibiting fungal invasion of peanut tissue was consistent, while others were variable. Some variation might be attributed to environmental influences, among other things, on the bacteria themselves. For example, plants treated with strain 520 (*Clavibacter michiganense*) had greater fungal invasion, compared to the control, at the first sampling date, but significantly less ($P < 0.10$) fungal invasion than the control at the second sampling date. Significant rainfall occurred between sampling dates. Fungal incidence in developing pegs and pods, and harvested seed, was consistently lower than the control with some bacteria, such as strain 539 (*Bacillus laterosporus*).

Peanut seed harvested from plants treated with bacterial strains have been assayed for aflatoxins. Seed from plants treated with those bacteria that most consistently reduced fungal invasion of developing peanut tissue, had reduced mean levels and variance of aflatoxins in seed compared to seed from control plants.

These studies indicate the potential for use of bacteria as biocontrol agents to minimize aflatoxins in peanuts. However, more studies need to be done on many aspects of these bacteria. Environmental influences on bacterial colonization and survival, during plant growth and development, seems to be critical. Studies on population dynamics of these bacteria are now underway, and will attempt to address some of these problems.

See also:

Mickler, C.J., K.L. Bowen and J.W. Kloepper. 1993. Development of a biological control assay and screening of selected geocarposphere bacteria against contamination of peanut by aflatoxigenic fungi. Phytopathology 83:(abstract, in press).

Bowen, K.L., J.W. Kloepper, H. Chourasia and C.J. Mickler. 1992. Selection of geocarposphere bacteria as candidate biological control agents for reducing aflatoxigenic fungi and aflatoxin contamination. Phytopathology 82:1121

AFLATOXIN WORKSHOP, OCTOBER 24-27, 1993

RELATIONSHIPS OF INOCULUM SOURCES OF ASPERGILLUS FLAVUS TO CONTROL BY DISEASE RESISTANCE AND MANAGEMENT IN CORN.

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Natural inoculum of *Aspergillus flavus* from waste corn was compared to artificial inoculation as a means of screening for resistance and determining resistance factors in the infection pathway on corn ears. Waste corn , naturally infested with *A. flavus*, was recovered from a corn bin in Iowa in 1993 and applied to field plots of four corn hybrids just before silking time at rates of 1, 5 and 10 kg/plot. Two of the hybrids had previously been determined by Dr. D. White, University of Illinois, to be resistant and two susceptible to *A. flavus*. All four hybrids also were planted at a location, 35 m from the other experiment, and artificially inoculated with a pin-bar device with a combined conidial suspension from three aflatoxin-producing isolates of *A. flavus*. In this experiment, kernel infection by *A. flavus* at harvest maturity was significantly lower in the resistant than in susceptible hybrids. In the first sampling of the natural infection experiment on August 19 , a strong correlation was obtained between rates of waste corn applied to plots and airborne inoculum of *A. flavus*. Also, infection of silks by *A. flavus* was detected in both susceptible hybrids but not in the resistant hybrids. These were promising results in determining the infection pattern of resistant and susceptible ears, but unfortunately, flooding in the plots due to excessive rains drastically reduced further spore development and release and the experiment was terminated prematurely. Results in 1992 had shown that, under more normal weather conditions, waste corn provides a reliable supply of airborne inoculum over several months and that inoculum levels are correlated with kernel infection. We conclude that waste corn could be a very successful natural infection screening method. It also has important advantages over artificial inoculation in that rates of inoculum are much easier to generate and it can account for resistance mechanisms on the pathway for infection of kernels. Application of this method into resistance studies will be a major component of our future program.

The average soilborne population of *A. flavus* in 40 fields in Iowa that had been surveyed in May and October in every year since 1988, showed an increase in population in July over that found in May. This pattern, which had previously been detected in a conservation tillage experiment in Nashua, Iowa, occurred again at that location in 1993, A model is proposed of the seasonal pattern of population development of *A. flavus* that incorporates weather and cultural factors. This suggests that a threshold value of the *A. flavus* population in July may be related to massive increases in population that occur under hot, dry conditions in epidemic years. If a mechanism such as sclerotial sporulation is determined to be involved in the population build up in spring and early summer, this may be amenable to control by management. Another opportunity for control may be in reduction of the July population below the critical threshold level. Further work will be directed at substantiating the model , then in evaluating control methods.

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Processing plant studies on fungi in pistachio nuts
A. Douglas King Jr. and Nelson Goodman

Studies in processing plants for control of aflatoxin in pistachio nuts were initiated in 1992 and continued in 1993. One consideration is the presence or absence of *Aspergillus flavus*, the mold producing aflatoxin. Data on pistachio processing had not been collected as in almonds and other tree nuts. Pistachio samples were collected from four processing plants during the autumn harvesting and processing season. The processing plants selected for sampling are representative of many processors and are situated to represent the crop geographically.

Fungal counts increased after harvest as the hulls were removed and through the flotation separation for split and complete shell pistachios (split shell nuts sink, intact shell nuts float). Fungal counts were reduced from a high of greater than 10,000 per gram to less than 1000 per gram by the drying process. Fungal counts from the four processors for dry sinkers and floaters varied dramatically. The highest counts were between 10,000 and 100,000 while the lowest were less than 100. The fungal count was indirectly related to the surface temperature of the kernels at the drier exit.

The incidence of *A. flavus* coming from the field was less than 5% of the kernels (similar to the data from last season). The incidence increased with hulling then dropped with the flotation separation step and associated washing. Kernel infection with *A. flavus* was 12% for dry sinkers and 19% for dry floaters. The reason for the increase during processing is unexplained.

Stem vascular tissue from in-shell nuts, from shelled kernels, and from stems of pistachio clusters were sometimes contaminated with mold growth. Endophytic molds were detected growing from the vascular tissue of fresh kernels, from the stem area of chlorine rinsed in-shell nuts and from stems that attach to the nuts. *Aspergillus niger, Cladosporium* spp., *Penicillium* spp. and *Rhizopus* spp. were most frequently identified growing from the plant stem part. *A. glaucus, A. flavus, A. ochraceus* and yeast were identified from less than 1% of the nuts. In-shell nuts without any endophytic molds were 48% of the total examined.

October 18, 1993

Summary: Panel Discussion on "Opportunities to Manipulate Microbial Ecology."

The panel consisted of: K. Bowen, J. Dorner, B. Horn, D. King, D. McGee, and I. Misaghi. The discussion was chaired by P. Cotty who also prepared this summary.

Several biological agents with potential to reduce aflatoxin contamination of various crops are under investigation. Misaghi and Bowen are investigating bacterial agents which grow well on crop components (cotton bolls and peanut pods, respectively) vulnerable to aflatoxin contamination. These agents act through colonizing ability, plant growth stimulation, and/or antibiotic production. Fungal agents capable of reducing contamination of peanuts were discussed by Dorner. These fungi are isolates of *A. flavus* and *A. parasiticus* which do not produce aflatoxins. Dorner's results suggest that application of multiple isolates may be more effective at preventing aflatoxin contamination of peanuts than application of individual isolates. The ecology and population dynamics of aflatoxin producing fungi during crop production and crop infection were examined by King and McGee. McGee's observations suggest that intervention to prevent *A. flavus* population increases and subsequent infection of corn may be most effective early in the season.

Biological agents are capable of active growth and reproduction. It was therefore questioned whether it would be sufficient to apply the agents only once or whether multiple applications would be necessary. Misaghi's field control results with a bacterium were achieved with multiple aerial applications. Dorner's control of aflatoxin contamination of peanut in environmental control plots was achieved with a single over-the-top granule application. The panel was split on whether the fungal agents would have to be applied each season and if the agents would have effects over multiple years. Some crops are typically rotated and thus a crop may only be in a field once in three years. This might reduce the possibility of additive effects over time. However, in experiments reported by Cotty in previous workshops, atoxigenic strains of *A. flavus* that had been applied to crops have been shown to overwinter between crop years. This suggests that benefits from an application might be realized in years subsequent to the year in which application was made. Thus, long term changes in fungal populations might be achieved. Cotty felt that large scale testing would be necessary to adequately assess long term effects of applications.

Several of the biological control agents being developed demonstrate potential as possible commercial products to be applied either to agricultural fields or to crops. Companies interested in commercializing such products often ask what the end user is willing to pay to prevent contamination. Since there are no products on the market used to prevent aflatoxin contamination, this is a difficult question to answer. Panel members and attendees had difficulty assessing the value a grower would place on controlling

contamination. It became apparent that the value would differ among crops, growing regions, and even between years.

Interest was also expressed in whether the biological control agents being investigated are active in untreated agricultural fields. If these agents are active, it was questioned if their activity partially explained the great variability seen in field plot experiments. The panel was again divided on this issue. Some felt the agents were not active in untreated fields. Others felt a low level of activity was present. Cotty mentioned that he and Bayman had isolated multiple *A. flavus* isolates from individual cotton bolls on several occasions and had found that bolls are often infected by multiple vegetative compatibility groups. Thus, he knew of cases where both toxigenic and atoxigenic strains had infected the same boll. Greenhouse experiments have shown that during co-infection, atoxigenic strains reduce contamination of cottonseed by the toxigenic strains.

PLATFORM PRESENTATIONS

Molecular Biology of A. flavus and Aflatoxin Formation

Aflatoxin Elimination: Understanding Genetic Regulation of Toxin Biosynthesis

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Aflatoxins B_1 and B_2 are secondary metabolites produced by the filamentous fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare that infect corn, cotton, peanut and tree nuts. These compounds are known to be toxic and carcinogenic to animals and present a potential threat to the health of human beings. In order to devise strategies for reducing or eliminating aflatoxin contamination from food and feed, extensive biochemical and genetic studies have been conducted by many researchers to better understand the molecular regulation of aflatoxin biosynthesis (see references). Pathway and regulatory genes have been cloned recently in our laboratory:

Pathway gene, *omt-1* Among the catalytic steps in the aflatoxin biosynthetic pathway, the conversion of sterigmatocystin to *O*-methylsterigmatocystin and the conversion of dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin are catalyzed by an S-adenosylmethionine-dependent O-methyltransferase. A cDNA library was constructed by using RNA isolated from a 24-h-old culture of wild-type *A. parasiticus* SRRC 143 and was screened by using polyclonal antiserum raised against a purified 40-kDa *O*-methyltransferase protein. A clone that harbored a full-length cDNA insert (1,460 bp) containing the 1,254-bp coding region of the gene, *omt*-1, was identified by the antiserum and isolated. The complete cDNA sequence was determined, and the corresponding 418-amino-acid sequence of the active enzyme with a molecular weight of 46,000 was deduced. The 1,460-bp *omt*-1 cDNA was cloned into an *Escherichia coli* expression system; a Western blot (immunoblot) analysis of crude extracts from this expression system revealed a 51-kDa fusion protein (fused with a 5-kDa β-galactosidase N-terminal fragment). Furthermore, enzymatic activity assays of the *E. coli* crude extracts showed that sterigmatocysin was converted to *O*-methylsterigmatocystin in the presence of *S*-adenosylmethionine. A 1.5-kb *omt*-1 gene transcript was detected by Northern (RNA) blot analysis in total RNAs isolated from submerged *A. parasiticus* cultures grown in a medium which induces aflatoxin B_1 production that were 24, 48, 72, and 96 h old but not in RNA from a culture that was 18 h old. Transcript accumulation correlated well with the increased rate of aflatoxin accumulation in these cultures.

Genomic DNA libraries were constructed in lambda EMBL3 using DNA isolated from wild type *A. parasiticus* SRRC 143 and in cosmid vector pAF1 using DNA isolated from aflatoxigenic *A. flavus* strain CRS01-2B. The genomic libraries were screened using *omt*-1 cDNA fragment from *A. parasiticus*. Positive clones were identified and the DNA fragments containing *omt*-1 gene from both *A. parasiticus* and *A. flavus* were subcloned and the completed sequences were determined. By comparison with the cDNA of the *omt*-1 gene, there were four introns in the coding region of the gene ranging from 52-bp to 60-bp in length. The putative S-adenosyl-methionine-binding motif proceeds immediately after the fourth intron. Comparison of the genomic DNA sequences of the *omt*-1 gene from *Aspergillus parasiticus* SRRC 143 and *A. flavus* strain CRA01-2B

showed that in the coding region they were the same in terms of the length of nucleotides, the number of introns, the position and the length of nucleotides in each intron, and the putative S-adenosyl-methionine-binding motif even though there are three substitutions in nucleotides. The DNA sequences between the two species are 97% identical in the coding region with only 38 nucleotide substitution within a total of 1,254 bp. Within the 418 amino acids in the methyltransferase protein, that the *omt*-1 gene encoded, only 14 amino acids were affected by the 38 nucleotide substitution between *A. flavus* and *A. parasiticus*.

Regulatory gene, *apa-2* (*afl-R*) An *Aspergillus parasiticus* gene, *apa-2* (now designated *afl-R*), was identified as a regulatory gene associated with aflatoxin biosynthesis. The *apa-2* gene was cloned on the basis of overproduction of pathway intermediates following transformation of fungal strains with cosmid DNA containing the aflatoxin biosynthetic genes *nor-1* and *ver-1*. Transformation of an *O*-methylsterigmatocystin-accumulating strain, *A. parasiticus* SRRC 2043, with a 5.5-kb *Hind*III-*Xba*I DNA fragment containing *apa-2* resulted in overproduction of all aflatoxin pathway intermediates analyzed. Specific enzyme activities associated with the conversion of norsolorinic acid and sterigmatocystin were increased approximately twofold. The *apa-2* gene also complemented an *A. flavus afl-2* mutant strain for aflatoxin production, suggesting that *apa-2* is functionally homologous to *afl-2* (see also G. A. Payne *et al.*). Comparison of the *A. parasiticus apa-2* gene DNA sequence with that of the *A. flavus afl-2* gene showed that they shared >95% DNA homology. Physical mapping of cosmid subclones placed *apa-2* approximately 8 kb from *ver-1* (see also J. Linz *et al.*).

The complete nucleotide sequence of the regulatory gene *afl-R* (*apa-2*) has been determined. The *afl-R* gene contains a single open reading frame encoding a protein of 376 amino acids and has a long untranslated sequence at its 3' end. The *afl-R* gene bears resemblance to other pathway-specific regulatory genes of ascomycetes. A putative DNA-binding domain of the GAL4-type binuclear zinc finger motif $Zn(II)_2Cys_6$, typical of regulatory genes, was identified. Northern hybridization analysis with aflatoxigenic *A. parasiticus* strains grown in nitrate medium indicates that the expression of the *afl-R* gene is required to turn on the transcription of three aflatoxin pathway structural genes, *nor-1*, *ver-1* and *omt-1*. This result indicates that a transcriptional activation mechanism is involved in the regulation of aflatoxin biosynthesis.

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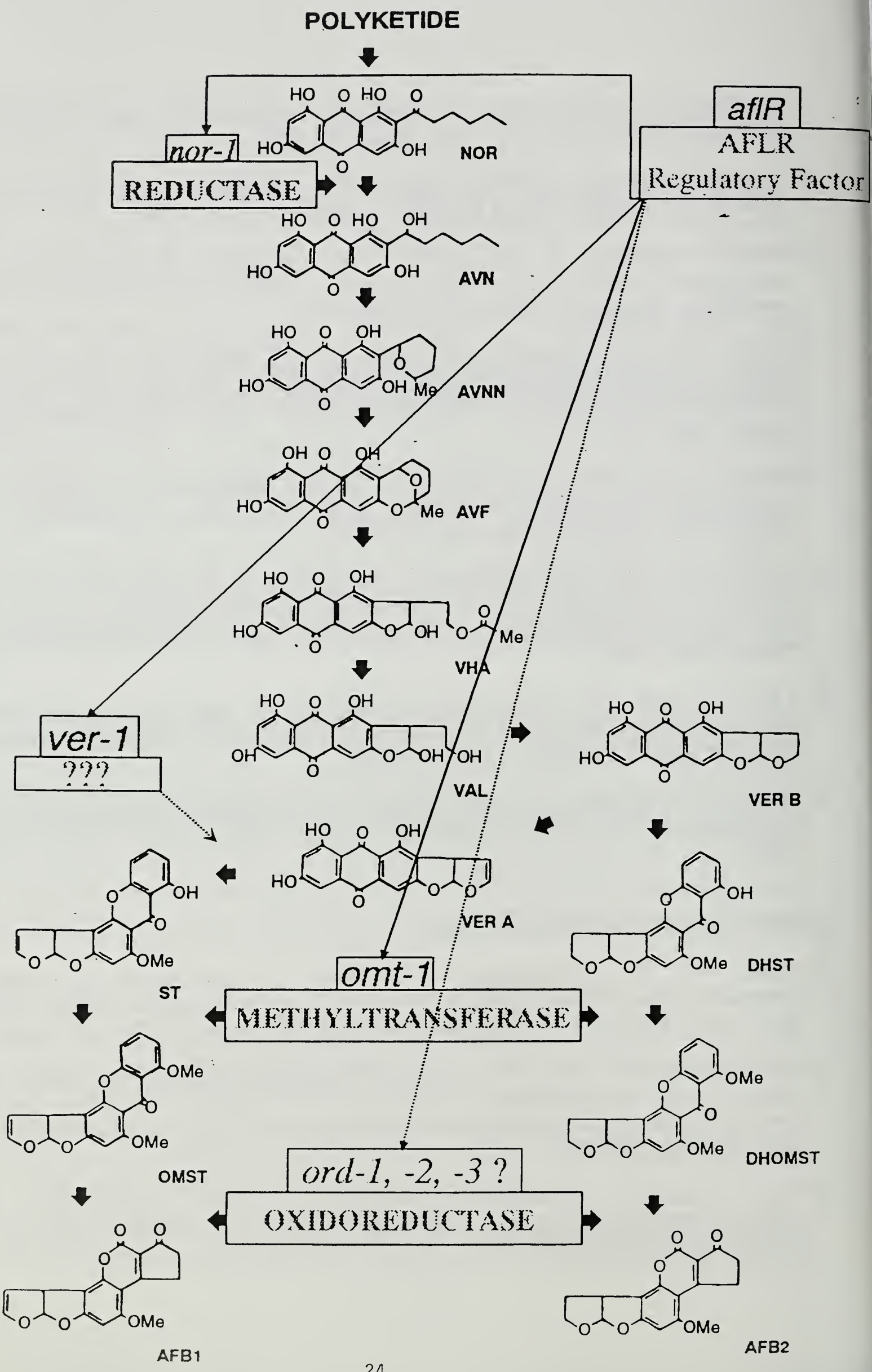
POLYKETIDE
nor-1
REDUCTASE
NOR
aflR
AFLR
Regulatory Factor
AVN
AVNN
AVF
VHA
ver-1
???
VAL
VER B
VER A
ST
omt-1
METHYLTRANSFERASE
DHST
OMST
DHOMST
ord-1, -2, -3 ?
OXIDOREDUCTASE
AFB1
AFB2

Molecular Approaches to Control Aflatoxin Contamination of Food Sources. G. A. Payne, Department of Plant Pathology, North Carolina State University, Raleigh NC and T. E. Cleveland and [2]D. Bhatnagar, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

A goal of my research program is to develop control strategies for the elimination of aflatoxin contamination. Currently we are focusing on the disruption of aflatoxin biosynthesis as a control mechanism. To achieve this goal, we are attempting to understand the factors that regulate aflatoxin biosynthesis. Biochemical evidence from our previous work (Payne et al, 1993) suggested that the *afl2* gene product (AFL2) has a regulatory role in aflatoxin biosynthesis in *Aspergillus flavus*. We have further characterized the *afl2* gene and have obtained conclusive evidence that this gene and its homologue in *A. parasiticus* (*apa2;* Chang et al 1993) regulate both early and late steps in aflatoxin biosynthesis. Thus, *afl2* and *apa2* appear to have a pivotal role in aflatoxin biosynthesis. This report covers our recent research in this area.

The *afl2* gene resides within a 2.7 kb fragment of DNA and codes for a transcript of 2.1 kb. A cDNA clone of 1.9 kb was sequenced, and the sequence was found to be identical to that of the genomic DNA, indicating that no introns occur over the transcribed region. Analysis of a cDNA clone revealed an open reading frame of 1300 nucleotides coding for 437 amino acids and putative protein of 46,679 daltons. Analysis of the amino acid sequence of the cDNA indicated that the polypeptide contains a zinc cluster DNA binding motif between amino acid position 29 and 49. This region contains the consensus amino acid sequence of Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys. This motif has been found in several transcriptional regulatory proteins. A perfect alignment of the cysteine residues was found with the GAL4 and PPR1 proteins from *Saccharomyces cerevisiae*, the LAC9 protein from *Kluyveromyces lactis*, and the *QA1F* protein from *Neurospora crassa.*

We also have evidence that the *afl2* gene codes for two overlapping divergently transcribed transcripts. This evidence comes from the analysis of a second cDNA library. In a second cDNA library we found several cDNA clones that were transcribed from the opposite strand of DNA. These clones were approximately 1.0 kb had an open reading frame of 702 bp. At present the function of the shorter transcript is unknown, but it is tempting to speculate that it may be involved in the regulation of the transcription of *afl2* itself. One could envision that media may influence the relative proportion of the two RNA species transcribed. It is interesting that one library was enriched in one species of transcript while the other library was enriched in the other type. In both cases RNA was prepared from cultures induced to produce aflatoxin by a nutritional shift; however, the procedure employed differed between the two experiments.

Now that we know that *afl2* regulates aflatoxin biosynthesis, we are interested in studying how it regulates the pathway. One approach is to study the effect of *afl2* on the expression of other pathway genes. To study gene expression, we have made reporter gene constructs. We have fused the GUS (β-glucuronidase) gene with the promoters of *nor1* and *ver1*, two other genes in the pathway. These constructs allow us to measure gene expression by the accumulation of a fluorescent compound rather than by transcript accumulation. We

have shown that the expression of GUS driven by the *nor1* and *ver1* promoters follows closely the accumulation of aflatoxin. Thus, these reporter constructs can be used to measure expression of these two genes. We are currently examining the effect of *afl2* on the expression of these two promoters in an experiment designed as follows. Strain 656-2, blocked at the *afl2* allele, was transformed with a *nor1*-GUS construct. The selectable marker, *pyr4*, was cotransformed with each reporter construct. Transformants were selected for uracil prototrophy and examined for aflatoxin production, GUS activity, and the presence of the GUS vector. As expected, none of the transformants from this first transformation that contained the nor-GUS construct produced GUS because they did not contain a functional *afl2* allele. One of these transformants was transformed a second time with a functional *afl2* allele and the gene that complements the *leu7* mutation. Transformants were selected for leucine prototrophy and assayed for aflatoxin and GUS activity. Of the 83 transformants assayed, 11 produced aflatoxin on coconut agar. Two of the aflatoxin producing colonies also produced GUS. Thus from this experiment we were able to demonstrate that a *nor1*-GUS construct is not expressed in a strain with a mutated *afl2*, but GUS expression can be induced by transformation of this same strain with a functional *afl2* gene. These data show that a functional *afl2* allele is required for the expression of the *nor1* gene. Additional studies are needed to further characterize the interrelationship between the expression of *afl2* and *nor1*. Studies are continuing to determine if AFL2 binds to the promoter regions of the *nor1* and *ver1* gene.

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IMMUNOCHEMICAL STUDIES ON THE ENZYMES OF AFLATOXIN BIOSYNTHESIS

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Attempts to produce both monoclonal (Mab) and polyclonal (Pab) antibodies against 3 key enzymes involved in aflatoxin (AF) biosynthesis, i.e. sterigmatocystin (ST) methyltransferase (ST-MTFase), norsolorinic acid reductase (NSR), and polyketide synthetase (PKS), were made. The objectives for these studies are: (1) to use the immunoaffinity method for purification of the enzymes, (2) to further our understanding on the structure and function of these enzymes, (3) immunochemical neutralization of the enzymes, and (4) to clone genes of these enzymes in collaboration with USDA scientists by supplying them with immunochemical reagents for enzyme characterization. During 1993, much of our efforts were devoted to the production and characterization of antibodies against ST-MTFase and NSR. Progress of this year's work follows:

(A) Studies on ST-methyltransferase:

1. Establishment of an effective new fluorometric method for ST-MTFase: An effective new spectrofluorometric method for ST-methyltransferase assay was established. The assay was carried out in a sepectrofluorometer with the excitation and emission wavelength set at 328 and 455 nm, respectively. The enzyme kinetics of MTFase was then calculated from the increase of fluorescence at 455 nm as the catalyzing the conversion of ST to OM-ST. The degree of the conversion efficiency was then calculated. This method was used throughout our study.

2. Production and characterization of polyclonal antibody against ST-MTFase: Polyclonal antibodies against the 40 kDa sterigmatocystin O-methyltransferase (ST-OMT) were produced after immunizing rabbits with the purified enzyme. Western blot analysis of the crude enzyme preparation from *Aspergillus parasiticus* strain 163 revealed one band corresponding to the ST-OMT (~ 40 kDa) as well as several other protein bands that didn't show any enzymatic activity. The antiserum was further purified by passing the ammonium sulfate precipitation-cut IgG through a column that was armed with proteins isolated from DEAE-Sephadex gel filtration fraction with immunoreactivity but containing no enzyme activity. Western blot analysis revealed that the purified antibodies, after the subtractive affinity chromatography, reacted primarily with one major (~ 40 kDa) and two minor protein bands of 40-46 kDa size proteins when large amounts of the crude extracts were used. At low protein concentration, only one immunoreactive band of 40 kDa was observed for the crude enzyme preparation. Using the purified antiserum, an indirect ELISA was established for the enzyme

detection. Analysis of various fungal extracts showed that the purified antiserum was highly specific for the enzyme.

3. Cross-reactivity of the antibodies against different fungi: Indirect ELISA and Western blot analysis revealed that the Pab obtained after DEAE column substraction was strongly reactive with the extracts from *Aspergillus parasiticus* that contain ST-MTFase of molecular size around 40 kDa. The antiserum also showed a weak reaction with the extracts obtained from *A. parasiticus* mutants, Nor1 and Ver, which have some leakage for aflatoxin production.. However, no cross-reaction was shown when extract obtained from *F. sporotrichioides* was tested.

4. Use of purified antibodies against ST-MTFase for cloning the enzyme gene: The purified antiserum against ST-MTFase has also been used successfully for screening the cDNA library in cloning the gene for the enzyme and in monitoring the enzyme produced in *Escherichia coli* in which this gene (omt-1) was expressed. Western blot analysis revealed that the protein prepared in the SRRC laboratory of USDA from *E. coli* with gene expression of vectors were also reacted with the partially purified antiserum and that the Pab only reacted with the extracts containing expression product of MTFase gene at a specific protein band.

5. Production of monoclonal antibodies against MT-MTFase: Efforts toward the production of monoclonal antibodies against MT-MTFase were made. Proteins of 40-42 kDa and 46 kDa molecular size of the partially purified enzyme preparations were eluted out from SDS-PAGE separately. These eluted proteins were then used as antigens respectively for monoclonal antibody production. Analysis of the antiserum form BABL/c mice with ELISA revealed that the antiserum from each group can recognize both the antigens (40-42 kDa and 46kDa). After fusion, a total of 23 cell lines elicited antibodies against 40-42 kDa was obtained. Further characterization and attempts to re-clone the specific cell line are undergoing. Preliminary data showed that two or 3 clones elicited monoclonal antibodies showing good specificity against ST-MTFase.

(B) Studies on norsolorinic acid reductase (NSR): In the last two years, we have successfully obtained both monoclonal and polyclonal antibodies against NSR reductase. Two hybridoma cell lines, namely ID9 and 10D2, were identified to edict monoclonal antibodies against NSR. The Mab elicited from 1D9 primarily reacted with the 48 kd protein band as well as a minor band of 43 kd size protein. In contrast, the Mab produced by clone 10D2 reacted with primarily the 43 kd protein with a minor reaction with the 48 kd proteins. Subsequently studies showed that antibody elicted from clone 10D2 primarly reacts with NSR. Thus much of this year's work was focused on the 10D2 Mab.

1. Production of monoclonal antibody using a hollow-fiber cell culture fermentor: Cell line 10D2 was adapted to grow in a serum-free medium (Amicon Division, W. R. Grace & Co.- Conn.) and then inoculated into a disposable

bioreactor (Amicon) connected to a circulation pump. The whole system was incubated in a 37°C room. The medium bottle was changed every 3 days, and 5-10mL of product sample was harvested every 1-2 days. The total run time was 40 days. A protocol for purification of IgG antibodies was established using HPLC equipped with an ABX column (Baker). The amount of antibody collected was analyzed by indirect ELISA coated with goat anti-mouse IgG antibodies and a series dilution of mouse IgG1 solution was used as standards. However, only small amounts of IgG1 (2 μg/mL), were produced under these conditions. This level was much lower than expected. Consequently, all the Mab used in the following studies was generated form ascites fluid of Balb/C mice using the 10D2 cells.

2. Neutralization of reductase activity by monoclonal antibodies: A series dilution of monoclonal antibodies 10D2, standard IgG1 antibodies and bovine serum albumin (BSA) were added separately into the reductase reaction mixture, and their effects on the reductase activity were analyzed by HPLC. Results indicated that BSA has no effect on the production of averantin, while both 10D2 and standard IgG antibodies inhibit the reductase activity. However, 10D2 antibodies have a much stronger inhibitory effect than the standard IgG, about 2-3 log cycle difference.

3. Electroelution and fragmentation of 43KD protein: Since there is no large quantity of 10D2 antibodies available for immunoaffinity purification of the 43KD protein, attempts were made to purify this protein using electroelution. The presence of 43KD band was monitored by Western blotting with 10D2 antibodies. The semi-purified fungal culture extract was first separated using preparative SDS-PAGE and three bands with molecular weights close to 43 KDa were cut and eluted out separately. Three bands were separated successfully and each elution product contained only one band on SDS-PAGE stained with Coomassie blue. One of the three bands was identified as the reductase by western blotting with 10D2 antibodies. We are currently attempting to map the reactive site of the enzyme with the 10D2 antibody. The reductase was subjected to trypsin digestion or cyanogen bromide cleavage, and the resulting fragments were further separated by HPLC with a C18 reverse phase column (Waters). The optimal conditions for fragmentation and subsequent HPLC separation are still under study. Peptide binding studies with 10D2 monoclonal antibodies using immunoblotings or indirect ELISA will be the next step.

4. Use of antibodies against NSR for cloning the enzyme gene: Both 1D9 and 10D2 Mabs are currently being used as tools for screening the cDNA library in cloning the gene for the enzyme. We are also using purified polyclonal antibodies as well as a sandwich ELISA method involving both Pab and Mabs for this purpose. We are working very closely with the scientists in the SRRC laboratory of USDA to clone this gene.

Structure/Function Analysis of an Aflatoxin Gene Cluster Isolated from Aspergillus parasiticus. N. Mahanti, F. Trail, R. Mehigh, T.S. Wu, R. Zhou, H.S. Liang, M. Rarick, J. Linz. Department of Food Science & Human Nutrition, Michigan State University, East Lansing, MI.

In the first two years of this project we were able to isolate two genes, ver-1 and nor-1, which are associated with the aflatoxin biosynthetic pathway. We studied the accumulation of transcripts from each of these two genes and observed a pattern of expression consistent with the onset of aflatoxin production which occurs late in growth (in laboratory shake culture). These two genes were found to be linked on one Aspergillus parasiticus chromosome. Two cosmids (norA and norB) containing overlapping chromosomal DNA fragments, were identified which carried the nor-1 gene only (norB) or the nor-1 and ver-1 genes (norA). We suspected that cosmid norA carried several other genes involved in aflatoxin biosynthesis.

3 specific objectives were outlined in the project for the 1993/1994 funding cycle.

1. Complete a physical/functional map of the aflatoxin gene cluster in A. parasiticus.

Structure of cosmid norA.

This cosmid contains approximately 36 kilobase pairs of A. parasiticus genomic DNA. A restriction map of this cosmid has been completed (using 4 different restriction endonucleases). In collaboration with the USDA ARS SRRC laboratory in New Orleans this entire region is undergoing nucleotide sequence analysis. It is anticipated that the sequence will be available within the next few months. Subcloned DNA fragments from cosmid norA were used to identify the location and pattern of expression of RNA transcripts arising from genes located on this cosmid. Besides nor-1 and ver-1, transcripts arising from 12 additional genes were identified in this analysis. Eleven of these genes are suspected of being involved in aflatoxin biosynthesis because their pattern of expression is similar to nor-1 and ver-1. Of these 11 genes, 3 genes adjacent to nor-1 are of particular interest. The transcripts arising from these genes are 7.0, 7.0 and 6.5 kilobases in size, consistent with the size expected for type I (large polyfunctional protein) polyketide synthetases. We have begun to characterize the function of these genes in "gene knockout" experiments (see below). The identification of polyketide synthetase activity in this biosynthetic pathway may be of particular significance because it is the first biochemical step and therefore a likely target of regulation.

2. Disrupt chromosomal copy of nor-1 and ver-1 genes associates with aflatoxin biosynthesis.

3. Subclone UVM 7/8 gene(s) - disrupt chromosomal copy of the genes.

Characterizing the function of cloned genes.

Using a recombinational inactivation strategy ("gene knockout"), we have initiated the study of the function of nor-1, ver-1, genes encoding the 3 large transcripts mentioned above, and another polyketide synthetase gene identified by hybridization to heterologous DNA probes (6 methyl salicylic acid synthetase from Penicillium patulum). To date, the nor-1 gene and a gene encoding one of the 7.0 kb transcripts (UVM8) have been successfully disrupted. Preliminary data suggest that the gene encoding the other 7.0 kb transcript has been successfully knocked out. Disruption constructs for each of the other genes of interest have

been constructed and are currently being utilized. The results of gene knockout (and nucleotide sequence data) suggest that nor-1 encodes a ketoreductase which is directly involved in aflatoxin biosynthesis (responsible for converting norsolorinic acid to averantin). Preliminary data suggest that when either of the two genes encoding the 7.0 kb transcripts is genetically disrupted, no intermediates in the aflatoxin pathway are detected, consistent with the hypothesis that these genes encode polyketide synthetase activities (or at least a very early step in the pathway).

Characterization of genomic clones identified by cDNA probes related to aflatoxin biosynthesis

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Genetics Department
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In a previous study we used a differential hybridization strategy to clone genes associated with aflatoxin biosynthesis in *Aspergillus parasiticus*. (Feng et al., 1992). A genomic library was screened with three different cDNA probes by a colony hybridization procedure. Nineteen clones were selected which were all positively correlated with and presumably enriched with aflatoxin related genes. Recently, we screened our 19 clone genomic library with a heterologous probe, a portion of the polyketide synthase (PKS) gene sequence, which is involved in the synthesis of the multifunctional 6-methyl- salicylic acid synthase (MSAS) of*Penicillium patulum*. Three of our genomic clones showed positive hybridization indicating the presence of similar PKS sequences. We choose one of these clones for molecular analysis. A genomic fragment 12 kilobases in size was completely sequenced and four exons were found by computer analysis, which showed an average of 57% amino acids similarity with 6-MSAS PKS.

The function of this PKS in *A. parasiticus* is not presently known. The Flavus group of Aspergillus, to which *A. parasiticus* belongs, has not been reported to produce the toxin patulin. Regardless of interpretations as to what function our *A. parasiticus* PKS contributes in this aflatoxigenic fungus, the facts will be determined by future experimentation. In subsequent experiments we plan to knock out this gene and determine its effect on aflatoxin formation. If disruption affects aflatoxin formation, then complementation transformation will be carried out with our cloned PKS to determine whether aflatoxin biosynthesis is restored. We also have several other candidates in our aflatoxin-related genomic library which also appear to carry PKS sequences judged by hybridization with the 6-MSAS probe. These clones will be similarly analyzed, their gene structure and coding products determined.

Panel Discussion Summary:

POTENTIAL CONTRIBUTIONS OF MOLECULAR BIOLOGY TO AFLATOXIN ELIMINATION

Panel Members: Thomas E. Cleveland, Fun-Sun Chu, Thomas Leonard, John E. Linz, Gary Payne, and Deepak Bhatnagar (Chair)

Aflatoxin synthesis has no obvious physiological role in primary growth and metabolism of the organism and, therefore, is considered to be a "secondary" process. As yet, there is no confirmed biological role of aflatoxin in the ecological survival of the fungal organism. However, since aflatoxins are toxic to certain potential competitor microbes in the ecosystem, a survival benefit to the producing fungi is implied. It should be noted, however, that aflatoxin per se is a poor antibiotic. It has been proposed that intensive agricultural practices are responsible for the creation of unique niches that under certain conditions select toxin-producing fungi. Contemporary crop production is based on intensive practices, and it is unlikely that these practices will be altered in a significant manner in the near future. It is, therefore, imperative to develop a comprehensive understanding of both the host-fungal interaction during aflatoxin contamination in various ecosystems and the molecular regulation of aflatoxin formation. This understanding may ultimately provide the tools for developing strategies for effective control of aflatoxigenic fungi and elimination of aflatoxin contamination from animal feed and human food chains. Several labs are actively involved in understanding the molecular regulation of aflatoxin biosynthesis. A comprehensive and cooperative research effort amongst these scientists has resulted in several discoveries in the last few years. A high degree of diligence, simultaneous use of multiple approaches, and the free exchange of research information and material between the members of the "Biosynthesis" group has resulted in accomplishments beyond anyone's imagination. The most recent of these accomplishments were outlined and discussed at the 1993 Aflatoxin Elimination Workshop. The following is a summary of the research findings:

Four major molecular biology approaches are being utilized by the researchers studying the elucidation of the aflatoxin biosynthetic pathway.

(1) <u>Enzyme→Gene</u>. The first approach involves isolation and purification to homogeneity of enzymes involved in aflatoxin biosynthesis. Using information obtained from the purified proteins, genes can be cloned for each function by established procedures. The advantage of this approach is that once an enzyme is purified, gene cloning is straightforward and the functionality of this gene is readily determined. The problem with this approach is that isolation and purification of intracellular fungal enzymes is extremely difficult; enzymes are present in low quantities, many are unstable, substrates are often insoluble, and the assay procedures are cumbersome. For these reasons, this approach was utilized by very few research groups. Recently a few labs, USDA/ARS/SRRC, J. Anderson (Texas), J. Linz (Michigan State Univ.) and K. Yabe (Japan) have begun to use this approach. The USDA lab has overcome several obstacles

to isolate at least three enzymes in the pathway. The use of molecular probes based on the purified protein sequence along with specific antibodies (from Dr. Chu's lab) has enabled Drs. Bhatnagar/Cleveland/Cary to clone a gene (*omt*) in the pathway based on one of these enzymes. With Dr. Chu's help, genes for the other two enzymes are also being cloned in the USDA lab.

(2) **Gene→Mutant→Protein**. The second approach being used to study aflatoxin biosynthesis is gene isolation by complementation for function that is lacking in a mutant strain. The benefit of this strategy is that many genes and their regulation can be identified very rapidly if pathway mutants are available. When this research began, no genes in the pathway had been isolated, and some scientists were skeptical that such a strategy would work for a secondary metabolite such as aflatoxin. The skepticism was the result of the many technical difficulties with this approach: an efficient genetic transformation system needed to be established and time consuming techniques to establish the specific function of the identified gene had to be developed. Drs. Payne and Linz, after several years of painstaking work established transformation systems for *A. flavus* and *A. parasiticus*, respectively. And using this strategy two pathway genes (*nor, ver*) and potentially a polyketide synthase gene (PKS) have been isolated from *A. parasiticus* by Dr. Linz and regulatory gene (*afl-2; A. flavus*) and (*apa-2; A. parasiticus*) have been isolated from the labs of Dr. Payne and Drs. Bhatnagar/ Cleveland. Further, Dr. Keller has also isolated a *ver* gene from *A. nidulans* that is identical to the *A. parasiticus ver* gene. Also, Drs. Linz and Keller, through a series of gene disruption studies, have established the function of the *nor* and *ver* genes, respectively. It has also been demonstrated in collaborative studies between these labs that all these genes reside on the same chromosome and that most, if not all aflatoxin pathway genes, are clustered on a 60-70 kb piece of DNA. It has also been demonstrated that the genomic organization of the pathway in the two fungi is very similar, but a few structural dissimilarities have also become evident.

(3) **Gene subtraction**. This approach involves obtaining a cDNA library encoding genes that are presumably involved only in secondary metabolic pathway because primary metabolic genes have been eliminated through gene subtraction. Using this methodology a number of genes can be cloned at one time. However, it is very difficult to know if a true subtraction of primary metabolic genes from secondary metabolic genes has been obtained, since there may be considerable overlap between primary and secondary metabolic processes; some primary metabolic genes could be involved in secondary processes. Also, the functions of genes identified by this approach are extremely difficult to establish. Dr. Leonard has recently isolated several cDNAs for transcripts induced during aflatoxin formation and has now sequenced parts of a PKS gene; the role of this gene in aflatoxin biosynthesis is yet to be determined.

(4) **Mapping of related genes**. In this approach, genes closely associated (temporally and spatially) with other identified pathway genes are selected for characteristics that resemble those of genes involved in aflatoxin biosynthesis. This approach has become possible only because a cluster of genes involved in aflatoxin biosynthesis has now been identified, and transcriptional mapping of this cluster has revealed other (than those already identified above) mRNA products whose temporal

expression resembles that of known aflatoxin pathway genes. The major problem with this approach is that the function (enzymatic or regulatory) of identified genes is very difficult and cumbersome to establish. Drs. Bhatnagar/Cleveland/Cary and Dr. Payne have utilized transcriptional mapping to identify and then sequence five other genes closely linked to previouly identified pathway genes in *A. flavus* and *A. parasiticus*, and these genes are thought to participate in toxin formation. Dr. Keller has also identified two genes in *A. nidulans* using the same approach. The role of these genes in the pathway is unknown although at least three of these genes are located on the gene cluster potentially responsible for aflatoxin biosynthesis.

What are the benefits of the molecular biology approach? The results of the Biosynthesis Group show that the approaches being used, in spite of all their shortcomings, have allowed us to isolate enzymes and genes in the biosynthetic pathway and study their regulation, thereby understanding how the fungus makes the toxin. Enough information is now available to devise a strategy to disrupt several large segments of the biosynthetic pathway. Drs. Linz and Keller have devised protocols to disrupt *nor* and *ver* genes, respectively, thereby creating "genetically engineered biocontrol strains." Since genes regulating aflatoxin biosynthesis have been identified by Drs. Payne/Bhatnagar/Cleveland, the effects of signals or compounds from plants or environment that either trigger or inhibit aflatoxin biosynthesis can now be monitored. Evidence is accumulating that compounds exist in crop plants that inhibit aflatoxin biosynthesis and a mechanism may exist in plants (so called resistant varieties that do not accumulate aflatoxin) to be able to turn off aflatoxin biosynthesis. There is increasing evidence that genes involved in aflatoxin biosynthesis may have some role to play in the survival of the fungus because sclerotia formation (Drs. Cotty/ Bhatnagar) and spore pigmentation (Dr. J. Salvo, GE) seem to be regulated in a manner similar to aflatoxin synthesis.

An immediate use of the findings is for the development of a gene-specific assay to screen plant genotypes. Such an assay would be based on the ability of plant extracts to inhibit or induce promoter activity of one of the pathway genes. Several strategies were discussed during the panel meeting to monitor such an activity. Ultimately it was conceded that the protocol developed by Dr. Payne and being tested at the USDA/SRRC lab was the best assay procedure. The protocol developed by Drs. Payne/Woloshuk involves measuring the promoter activity of pathway gene by fusing the promoter to a reporter gene such as GUS. The expression of GUS activity during aflatoxin elaboration has been measured by the production of an easily visualized compound in corn kernels. The advantages of such an assay have been demonstrated to be: 1) it is easier and safer than aflatoxin analyses, 2) it is more sensitive and less variable because it directly measures a gene in the pathway, and 3) it will allow the identification of compounds that block the early, middle or late steps in the pathway (since genes have now been cloned from various stages in the pathway).

In conclusion, the panelists agreed that the disruption of aflatoxin biosynthesis as a strategy for aflatoxin elimination was a sound approach. This strategy along with a strategy to effectively and promptly monitor resistance against the fungus could assist in the elimination of aflatoxin accumulation in plants.

Potential Contributions of Molecular Biology to Aflatoxin Elimination

Three major molecular biology approaches in understanding aflatoxin biosynthesis:

(1) Enzyme ⟶ Gene

(2) Gene ⟷ Protein

(3) Gene subtraction

(1) Enzyme ⟶ Gene

D. Bhatnagar / T. Cleveland / J. Cary (SRRC)
F.S. Chu (Wisconsin)

J. Anderson (Texas) / J. Linz (MSU)

K. Yabe (Japan)

Problems: Isolation and Purification of enzyme is a tall order

Benefits: Gene cloning straight forward and function of the gene is known

(2) Gene $\xrightarrow{\text{Mutant}}$ Protein

G. Payne (NC State)
J. Linz (MSU)
D. Bhatnagar / T. Cleveland (SRRC)
N. Keller / T. Adams (Texas A&M)

F.S. Chu (Wisconsin)

Difficulties:

a) establishing a genetic transformation system
b) function of gene is time consuming to establish

Benefits:

can identify several genes and regulation very rapidly

(3) Gene Subtraction

i.e. Separation
- Primary metabolic genes
- Secondary metabolic genes

T. Leonard (Univ of Wisconsin)
G. Payne (NC State)
M. Wright (SRRC)

Difficulties:

a) not known if true subtraction is attained
b) what if primary genes are involved in secondary processes
c) functions of genes extremely difficult to establish

Benefits: can clone a number of genes at one time

Benefits of the Molecular Biology Approach:

(1) Understand how and why the fungus makes the toxin
    i.e. determine its regulation
(2) Can monitor what signals (plants/environment)
    trigger the aflatoxin biosynthetic process
(3) Can estimate if survival of fungus is affected by
    genes involved in aflatoxin biosynthesis
    a) sclerotia formation
    b) spore pigmentation (J. Salvo, GE)

PLATFORM PRESENTATIONS

Crop Resistance – Identification and Utilization

TRANSFORMATION OF PEANUT FOR ENHANCED RESISTANCE TO *ASPERGILLUS FLAVUS*

A. Weissinger[1], L. Urban[1], R. Boston[1], W. N. Zuo[1],
S. D. Utomo[1], G. Menon[1], P. Ozias-Akins[2], T. E. Cleveland[3]

We have developed a rudimentary system for the transfer of alien genes into peanut via microprojectile bombardment of embryogenic cultures (Ozias-Akins, et al., in press). We are also working both to improve the efficiency of this system, and to develop *Agrobacterium*-based protocols which are expected to replace microprojectile bombardment as the method of choice for peanut transformation. Concurrently with these efforts, we have begun work to introduce genes with demonstrated anti-fungal activity into peanut using the most advanced biolistic protocols.

Our first attempts involved the introduction of a chimeric gene encoding a basic chitinase from common bean (Broglie, et al., 1991). Transformation has been accomplished via the biolistic process in other systems by the expedient of co-bombardment, in which a plasmid with the gene of interest is mixed with another carrying a selectable marker gene. This system is desirable, since it simplifies construction of transformation vectors, and because it typically leads to integration of the selectable marker gene at a chromosomal site remote from that at which the gene of interest is inserted. Numerous attempts at cobombardment of peanut embryogenic cultures with p35SCHN 641 (chitinase; 11.5 kb) and pH602 (HPH=Hygromycin resistance; 13.5 kb), however, have thus far resulted in the recovery of transformed tissues which carry only the selectable marker. Because the bean chitinase is routinely expressed in common bean, and has been expressed successfully in other heterologous systems, it is unlikely that its expression in peanut is resulting in loss of viability. Rather, it appears likely that cotransformation is very inefficient in this system, and thus probability of recovering cotransformants is low. We have therefore reengineered the chitinase gene by selective PCR amplification of the 981 bp chitinase coding sequence from the 3.5 kb fragment contained in the original construct. This was subsequently introduced into a derivative of pH602, to produce the plasmid pBluescript-CHN-Hygro (chitinase and HPH, both driven by 35S promoters; 7.2 kb). Importantly, this plasmid allows the delivery of both genes as a single covalently-linked unit, and also permits the delivery of more than three times as many copies of each gene to be delivered during bombardment as was possible with the combination of two plasmids. These modifications should allow the recovery of chitinase transgenics, and should also increase overall transformation efficiency.

Another gene with potential activity against *Aspergillus* and other fungi is a maize Ribosome Inactivating Protein (RIP). RIP functions to depurinate a sequence-specific loop on alien ribosomes, preventing the binding of a critical translation factor, effectively blocking protein synthesis in the target organism. This product is expressed in corn in an inactive pro-RIP form which is subsequently activated by proteolytic cleavage. We have successfully engineered this gene to produce a product which is active without the need for processing. In order to test the efficacy of this gene product in vivo as quickly as possible, both the pro-RIP and the modified form of the gene have been transformed into tobacco. Importantly, expression of the RIP in its active form had no adverse effect on the growth and development of tobacco plants. These plants are now being propagated vegetatively for use in efficacy trials against *Aspergillus* and other pathogenic fungi. If successful, the method of early efficacy testing in transgenic tobaccos will be adopted for the testing of other gene products as well. The use of vegetative propagules may also be useful in peanut, since it would permit rapid screening of genotypes against multiple pathogens, without jeopardizing the reproductive capacity of the primary transformant.

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TRANSFORMATION SYSTEMS FOR PEANUT - EVALUATION OF ALTERNATIVES

P. Ozias-Akins[1], R. Gill[1], W.F. Anderson[1], C. Singsit[1], and A.K. Weissinger[2]

A method for introducing foreign genes into peanut has been developed (Ozias-Akins et al., 1993, Plant Science, in press); however, refinement of the technique and exploration of alternative methods could lead to the enhancement of transformation frequencies and reduction in the amount of time required to obtain transgenic plants. Integration patterns and transmission of foreign genes to progeny are questions that have not yet been addressed but which could be affected by transformation method.

The system we have developed for transformation of embryogenic callus is reliable and reproducible. Somatic embryos/embryogenic callus are bombarded with DNA containing a gene conferring resistance to the antibiotic hygromycin. Stably transformed callus/embryos can be obtained through selection on media containing hygromycin. Rapidly growing, solid transgenic cell lines appear 2-6 months after the initiation of selection. Incorporation of foreign DNA into the genome of regenerated plants has been verified by the polymerase chain reaction and Southern blot analysis. The transgenic cell lines can be maintained under selection on hygromycin, and regeneration can be initiated. This method, although lengthy, is likely to produce non-chimeric regenerated plants.

A natural DNA delivery system, *Agrobacterium tumefaciens*, also can be used to produce transgenic cell lines when *Agrobacterium* is cocultivated with embryogenic callus. Based on the first series of experiments with three genotypes and a single strain of *Agrobacterium*, the frequency of transformation does not appear to be significantly higher than what we have observed with microprojectile bombardment, perhaps due to the slow recovery of tissue after cocultivation. Extensive Southern blot analysis will be carried out on regenerated plants to determine if patterns of DNA integration are substantially different from bombarded transformants.

Cotransformation of cells with a selectable marker gene on one plasmid and a gene of interest on a separate plasmid would be desirable in order to reduce the effort required to produce plasmid constructs containing both selectable marker and desirable genes. If the unlinked genes were integrated at unlinked loci, it also would provide the opportunity to genetically separate the two genes and remove the antibiotic resistance gene from material with commercial potential. Unfortunately, we have obtained no evidence for cotransformation in 26 cell lines that were selected for hygromycin resistance and presence of the hygromycin resistance gene.

Based on our cotransformation data, reengineering of plasmids will be required in order to combine putative fungal resistance genes with hygromycin resistance. A construct containing the selectable marker gene and a synthetic Bt toxin gene has been used in several bombardment experiments. Four hygromycin resistant cell lines from the first experiment have been isolated and all contain the Bt gene as demonstrated by PCR. Within a year we should have information on the efficacy of plant-produced Bt toxin for elimination of lesser cornstalk borer damage to peanut.

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Control of Aspergillus flavus infection and/or aflatoxin biosynthesis using host and non-host genes. T. Cleveland, J. Cary, R. Brown, T. Jacks, H. Zeringue, J. Neucere, D. Bhatnagar, USDA, ARS, New Orleans; G. Payne, C. Woloshuk, A. Weissinger, North Carolina State Univ.; P. Ozias-Akins, Univ. Georgia; S. Tuzun, P. Gay, Auburn University; C. Chlan, Univ. Southwestern LA; B. Guo, J. Russin, Louisiana State Univ.; J. Jaynes, Demeter Biotechnologies Inc.

Genes that could be manipulated to control A. flavus and/or aflatoxin expression may exist in host crops (cottonseed, corn, peanut, and treenuts) subject to aflatoxin contamination ("host" genes) or from other sources ("non-host" genes) such as from plant species not infected by A. flavus, microbes and even animals. For example, synthetic analogues of cecropin, an insect peptide with antimicrobial properties, inhibited A. flavus growth (at a level of 10^{-5} M). Synthetic genes based on the amino acid sequence of cecropin analogues are being produced for genetic engineering of cotton and peanut for resistance to A. flavus.

Resistance traits in corn and cotton have been identified that inhibit growth of A. flavus and/or aflatoxin contamination. Antifungal volatile compounds (alkenals and alkanals) probably the products of lipoxygenase reactions were produced by infected cotton bolls and corn kernel tissues. Lipoxygenase genes have been cloned and could be used in plant genetic engineering to improve native resistance against A. flavus. Lipoxygenase is a potential marker for selection of resistant plant varieties during plant breeding.

Corn varieties with varying levels of resistance to A. flavus were assayed for chitinases (potentially lytic to fungal cells); no correlation was found between corn kernel chitinases and fungal resistance. Bacteria previously shown to secrete large molecular weight activity(ies) that inhibit growth of A. flavus, produced chitinases in culture which are being tested for antifungal properties; bacterial chitinase genes are being cloned for possible use in transformation of cotton and peanut.

Genetically engineered A. flavus strains containing various promoter-"reporter" gene constructs were used to monitor fungal growth and aflatoxin gene expression individually in fruit/seed tissues of inoculated cotton bolls and corn kernels. Using the reporter gene assay to monitor fungal growth, one variety (MAS) of corn, which possesses field resistance to aflatoxin contamination, showed significant resistance to embryo invasion by A. flavus and much less aflatoxin contamination relative to other corn varieties. A. flavus strains containing reporter genes should be useful in identifying host plant traits affecting aflatoxin expression in situ.

Treatments of corn kernels with chloroform to remove waxes and cutinase to remove the cutin layer from the pericarp followed by inoculation with A. flavus, resulted in significantly higher aflatoxin in the MAS variety, thus demonstrating the importance of the cutin layer of kernels as a structural barrier to invasion by A. flavus. However, significant resistance to aflatoxin contamination was maintained in the MAS variety relative to other varieties even in MAS kernels that were wound-inoculated to bypass outer kernel structural barriers, thus suggesting that the MAS kernels, if not damaged too severely, have chemical resistance to aflatoxin contamination. Once traits (host or non-host) which reduce aflatoxin expression in plants are identified, genes for these traits will be cloned and used to genetically engineer crops (cotton, corn, peanuts and tree nuts) for resistance to aflatoxin contamination.

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Title: Transformation and Regeneration of Cotton to Yield Improved Resistance to A. flavus

Authors: Caryl A. Chlan, Lin Junmin, Laura Willis, Rachel LaPorte, and Sheryl Kunning (Biology Department, The University of Southwestern Louisiana, Lafayette, LA 70504) and Jeffrey Cary and Thomas E. Cleveland (Southern Regional Research Laboratory, USDA/ARS, New Orleans, LA 70179)

Our goal in this project is to transform and regenerate cotton plants that have been genetically engineered in the laboratory to have increased resistance to fungal pathogens. Specifically, we are interested in engineering cotton so that it will have increased resistance to the fungal pathogen, Aspergillus flavus. Although the primary product of the cotton industry is cotton fiber, cottonseed is an important byproduct. The monetary value of cottonseed used as feed is affected by the levels of aflatoxin contamination. Although this problem has been studied for many years, conventional control measures have not been effective, and strains of cotton that are naturally resistant to A. flavus are not available. Genetic engineering of cotton using molecular techniques is a logical approach to this problem.

To genetically engineer and regenerate cotton, we have employed two approaches. We have performed numerous tests using genetically engineered Agrobacterium strains to introduce new genes into cotton. We have engineered three test Agrobacterium strains with gene constructs to use in this study, we have not yet successfully regenerated transformed plants from cotton hypocotyl tissues treated with these strains. We have, however, successfully regenerated cotton plants from non-transformed cotton hypocotyl tissues. It appears that in our transformation studies, treatment of the hypocotyl sections with Agrobacterium and subsequently with antibiotics, retards the growth of the tissue. In an effort to speed up the regeneration process following treatment with Agrobacterium, we are in the process of transforming embryogenic suspension cell cultures.

We have recently implemented a second method for introducing foreign genes into cotton. This method involves the transfer of DNA into plant cells by bombardment with DNA coated particles. One of the major advantages of this technique is that the transfer is not mediated by Agrobacterium, and tissue damage is minimized. We have optimized the biolistic system for transplant viability and transient expression using a GUS reporter gene. The optimized conditions have been used to treat over 500 meristem explant sections. These sections are being screened for the presence of GUS activity.

Both the biolistic system, and transformation of suspension cell cultures will be used to introduce genes that may confer increased resistance to fungal pathogens. This laboratory has been able to prepare two clones that contain promoters that are specific for expression in the cotton seed. These promoters will be linked to structural genes that may confer increased resistance to pathogens, and then be used to transform cotton tissue. Other potentially useful promoter elements and structural genes may be isolated from a cotton leaf genomic DNA library that we constructed this year in the lambda-dash vector. We are currently screening this library for promoters of constitutively expressed gene promoter elements. These promoters and structural genes will be used to transform cotton tissue using both transformation/regeneration protocols to engineer cotton to confer increased resistance to fungal pathogens.

INSERTION OF CHITIN-BINDING GENES IN WALNUT AND BIOASSAYS WITH ASPERGILLUS FLAVUS

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Aflatoxin contamination following infection by the fungus Aspergillus flavus Link is a serious problem in many grain crops and tree nuts. So far, the strategies used to reduce contamination have essentially dealt with preventive crop management techniques. Another approach involves insertion of fungus growth inhibiting genes, such as the chitin-binding genes. The previous development of an efficient transformation system in walnuts enabled us to test the feasibility of controlling A. flavus through genetic engineering.

Seven different gene constructs (barley lectin sense and antisense, hevein sense and antisense, stinging nettle lectin sense and antisense, and unmodified pMON 893, in Agrobacterium tumefaciens strain ABI or LB 4404 were used to transform 4 different walnut embryo lines ('SU2', 'Pedro x 56-224 #3', '85-8 x 85-10 #1', 'Chandler x 85-10 #1'). A total of 11 transformed lines, all originating from the SU2 line, were isolated and gene insertion was confirmed by Southern blot analysis. Protein was also detected by Western blot analysis for the transformed barley lines.

Activity against A. flavus was determined on inoculated embryos for 8 of the transformed lines using 2 different approaches. In the first one, aflatoxin was assayed every 24 hour, for 4 consecutive days, after a 4 day incubation in the dark at 30 C and 80% RH. In the second approach, the amount of sporulation of A. flavus was quantified by plating, every 12 hours for 2 consecutive days, aliquots of spore suspensions obtained from fungal growth on embryo tissues after a 2 to 2.5 day incubation in the dark at 30 C and 80% RH.

Because aflatoxin production on non transformed embryos was erratic, it was not possible to use this method for testing the activity of the different chitin-binding genes against A. flavus. When sporulation over time was used, no significant difference was detected between the transformed lines and the control line SU2, or transformed lines appeared to provide a better substrate for fungal growth. Future work will focus on developing improved bioassays for detection of activity against the fungus, and in selecting genes known to inhibit A. flavus.

Opportunities and Obstacles in a Genetic Engineering Approach - Including Reaching the Fungus during Critical Plant Development Stages

Panel: Dr. Bill Anderson, Dr. Caryl A. Chlan (chair), Dr. Thomas E. Cleveland, Ms. Sylvaine Coulibaly, Dr. Gayle McGranahan, Dr. Peggy Ozias-Akins, Dr. Art Weissinger

This panel discussion focused on general approaches to confer resistance to *A. flavus*. There were three general areas of discussion: ideas, suggestions, and possible approaches for blocking infection of plant tissue by *A. flavus* through genetic engineering, methods of identifying and characterizing genes that may confer resistance to *A. flavus*, and problems associated with regenerating viable plants.

One possible approach for blocking the infection of plants discussed during this session involved induction of natural defense genes by compounds such as salicylic acid. Salicylic acid has been show to be an effective inducer of multiple defenses in curcurbits and the solanaceae. However, defense chemicals induced by this mechanism do not accumulate in the seed where aflatoxin contamination occurs, thus casting some doubt about the efficacy of this approach.

The panel did agree that multiple genes will probably be necessary to engineer plants that are resistant to *A. flavus*, but there was no consensus as to which genes have the greatest probability for success. Introduction of multiple genes to confer resistance to *A. flavus* would decrease the probability that *A. flavus* would readily develop resistance to those anti-fungal compounds. Several genes have been identified as possible candidates - chitinases, ribosome inactivating proteins, chitin binding proteins, peroxidases, insecticidal proteins (BT toxin), thionin and osmotin among others. However, each of these gene products should be tested for efficacy in inhibiting the fungus prior to introduction into plants, and the subsequent time consuming regeneration process.

Once plants have been transformed with new genes, it is necessary to regenerate viable plants from the transformed tissue. There was some discussion concerning the apparent recalcitrance of transformed peanut tissue to flower and set seed. One possible explanation is that the problems with delayed and infrequent flowering in peanut are the result of antibiotic selection for transformed tissue. This problem may be genotype specific. However, the transformed plants are now flowering, and have begun to develop pegs.

The transformation and regeneration of transformed plant tissue has progressed rapidly for all three crops discussed - cotton, walnut and peanut. Although corn can be genetically engineered and regenerated, it is a very expensive process, and private industry has already initiated experiments to generate genetically improved corn, however, engineering a variety of aflatoxin resistant corn does not appear to be a high priority. Since genes can be introduced into cells, and cell regenerated into plants, the remaining obstacles pertain to which gene should be introduced, and how to manipulate those genes to express the anti-fungal compounds in appropriate tissues at levels that will be effective. This will be dependent upon fusion of structural genes to promoter elements that will confer the appropriate tissue specificity and developmentally regulated expression.

PLATFORM PRESENTATIONS

Crop Resistance - Conventional Breeding

Aflatoxin Elimination Workshop
Little Rock, Arkansas, October 24-26, 1993

EVALUATION OF COMMERCIAL MAIZE GENOTYPES FOR RESISTANCE TO AFLATOXIN CONTAMINATION OF THE WHOLE GRAIN.

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The initial objective of this cooperative research, to identify sources of genetic resistance among maize inbreds and hybrids, is completed. Thirty-four (34) commercial Corn Belt hybrids and twenty-five (25) elite inbreds were evaluated for their resistance/susceptibility to aflatoxin contamination of the grain by Aspergillus flavus at Bloomington, IL and Union City, TN. Maize ears in the late-milk to early-dough stage of kernel maturity are wound-inoculated with A. flavus. Approximately twenty (20) non-damaged whole kernels surrounding each wound-inoculation site are removed from the ear. Elimination of the individual wound-inoculated kernels from our sample removes a source of highly aflatoxin-contaminated grain that has little relevance to the resistance of maturing grain to A. flavus infection and subsequent aflatoxin contamination. Climatological patterns at Bloomington, IL allowed contrasts between a 'typical' corn production year (1990) and a year of extended drought, but without unusually high temperatures (1991). Contrasts for Union City included typically hot (1990) versus cool (1992) growing conditions, the latter being characteristic of the upper Corn Belt. Under conditions of drought or temperature stress, specific inbreds improved the aflatoxin resistance of a hybrid while other inbreds, particularly B73, were associated with increased aflatoxin contamination of the grain. The hybrid ranked first overall in aflatoxin resistance [CIBA Seeds No. 00544 x 00565], was contaminated with substantially less aflatoxin (25 ppb, 30 ppb, 56 ppb, 240 ppb) than individual hybrids ranked last in each field trial (430 ppb, 590 ppb, 640 ppb & 1870 ppb). Temperature stress (Union City, 1990) was associated with a several-fold increase in the level of aflatoxin contamination among some of the hybrids. The interpretation of aflatoxin resistance rank is not confounded by locational or yearly differences in either (1) the aflatoxin-producing ability of the A. flavus population, (2) the production of an infective conidial inoculum, (3) the dispersal of infective inoculum to the maturing ears, or (4) the extent of insect damage to the ears. In 1993 several of our best and worst performing hybrids and inbreds were planted late near Weslaco, TX to assess their performance in a 'worst possible case scenario' high temperature stress environment.

As part of our Cooperative Research and Development Agreement No. 58-3K95-M-67, several new experiments were initiated in 1993 (1) to evaluate the ability of our screening procedure to distinguish among individual ears of resistant versus susceptible hybrids as required for RFLP mapping; (2) to determine if pollen source influences aflatoxin resistance; (3) to investigate the potential association between heritable traits of kernel splitting/seed coat tearing and aflatoxin susceptibility; (4) Dr. R.A. Norton, Mycotoxin Research Unit, NCAUR, has begun to examine these 'resistant' versus 'susceptible' inbreds and hybrids for allelochemicals impacting A. flavus growth or aflatoxin production at different stages of kernel maturation.

Resistance to Aspergillus Ear Rot and Aflatoxin Production

K.W. Campbell, D.G. White, and T.R. Rocheford University of Illinois

In 1991 crosses of 1,189 corn inbreds with Mo17 and 978 corn inbreds with B73 were evaluated for *A. flavus* ear rot, using an inoculator developed at the University of Illinois. From the F_1 crosses 18 inbreds crossed with B73 and 17 inbreds crossed with Mo17 were selected (35 total inbreds) for further study.

In 1992, the parental inbreds, F_1, F_2 and backcross to the susceptible parent generations were evaluated. F_3 generations were also evaluated for ten of the inbreds. Data were analyzed using generation mean analysis to determine the mode of inheritance for each of the 35. Analyses showed four of the resistant inbreds had dominance. In 1992, aflatoxin analysis was completed on parental inbreds, and subsequent F_1 and F_2 generations. Aflatoxin analyses were completed by Eli Glendloff at the University of Wisconsin. The range of aflatoxin values were: 0 - 4,000+, 0 - 3,983 and 4 - 1,814 ppb/aflatoxin B_1 for the self pollinated F_2's, inbreds, and F_1's respectively. Inbreds or F_1's were also evaluated by Neil Widstrom, USDA/ARS, Tifton, Georgia, Gene Scott and Natale Zummo USDA/ARS Starkville, Mississippi, and Bob Brown and Ed Cleveland USDA/ARS New Orleans, Louisiana. From a compilation of these results and the Illinois results, 10 inbreds were selected for further study.

In 1993 we are evaluating 35 F_1 crosses, including additional germ plasm not previously evaluated. With the ten most promising sources of resistances from 1992 studies we are evaluating both inbred parents, the F_1, both backcross generations, F_2, F_3, and in most cases F_4 and backcross susceptible self generations. We have completed about two-thirds of the ear rot ratings in the field at this time. We will do aflatoxin analysis on the parental inbreds, F_1, F_3, and F_4 of most resistant sources.

Restriction fragment length polymorphism (RFLP) analyses were performed on 14 of the better sources of resistance. The purpose was to identify RFLP probes polymorphic between the sources of resistance and B73 or Mo17. RFLP mapping populations have been developed by crossing some of the sources of resistance with B73 followed by selfing to produce F_3 families. Over 100 probes were analyzed and very high levels of polymorphism were detected between the sources of resistance and B73. This indicates RFLP mapping to identify chromosomal regions associated with resistance to *A. flavus* will not be limited by inability to detect polymorphism throughout the maize genome.

RFLP mapping analyses of F_3 families developed from the sources of resistance LB31 and R001-75 crossed with B73 are underway. Approximately ten probes have been evaluated on 100 F_3 families developed from each of the crosses. The 100 F_3 families were grown, inoculated and evaluated in replicated trials in 1992 and 1993. The probe information has been evaluated relative to the field data just from 1992 since we do not have 1993 data yet. No significant associations have been detected thus far. However, this is not surprising because we only have one year's field data and we currently only have a small portion of the maize genome covered. We intend to put more than 70 probes on the F_3 families this winter and analyze them with the combined 1992 and 1993 data. Analysis of the RFLP patterns of LB31 and R001-75 in comparison to Mo17 and B73 revealed that neither is significantly closer to Mo17 or B73 in terms of common RFLP fragments. This suggests that these sources of resistance could be introgressed to either side of a maize heterotic pattern.

Campbell, K.W., D.G. White, J. Toman Jr., and T.R. Rocheford. 1993. Sources of resistance in F_1 corn hybrids to ear rot caused by *Aspergillus flavus*. Plant Dis: In press.

Breeding Peanut for Resistance to Pre-harvest Aflatoxin Contamination. C. C. Holbrook[1], D. M. Wilson[2], M. E. Matheron[3] and W. F. Anderson[1]. [1]USDA-ARS, Tifton, GA; [2]Univ. of Georgia, Tifton, GA; [3]Univ. of Arizona, Somerton, AZ.

Pre-harvest aflatoxin contamination is the most serious challenge facing the U. S. peanut industry. In 1990 a research project was initiated to attempt to develop genetic resistance to PAC in peanut. The first objective of this project was to develop large scale screening techniques which could be used to evaluate germplasm for resistance. Reliable techniques have been developed to allow for large scale field screening at Tifton, GA and Yuma, AZ. The second objective was to select a core collection for the U.S. germplasm collection. This objective has been achieved. In addition, the core collection approach has been verified using data on leafspot resistance for the entire collection. The third objective was to examine the core collection for resistance to PAC. The majority of the accessions in the core collection were evaluated in 1991 and 1992. The remaining accession are under testing in 1993. Any accession which appears promising in the first year of testing is then reevaluated for a second year. Through 1992 nine accessions have been reevaluated and one (232CC = P.I.290626) continued to show an aflatoxin level < 20% of the check over three environments. An additional accession (234CC = P.I.159664) also demonstrated a high level of resistance to PAC in two of three environments.

Southern Runner Preharvest Aflatoxin Resistance Correlated with Drought Tolerance

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The development of absolute resistance to preharvest *Aspergillus flavus/A. parasiticus* invasion and aflatoxin contamination may not be possible using conventional breeding approaches. However, recent studies have demonstrated that selected germplasm differ in the degree of susceptibility/resistance to preharvest aflatoxin contamination. Data from a 1990 field study indicated that the Southern Runner variety had some resistance to preharvest aflatoxin contamination. This prompted comparative studies between the Florunner and Southern Runner cultivars (1991) and Florunner, Southern Runner and a Brazilian accession (1992). Florunner and the Brazilian accession are the parents of the Southern variety. It was concluded from these studies that the Southern Runner variety was more resistant to preharvest aflatoxin contamination than the Florunner variety. Furthermore, this resistance was inherited from the Brazilian parent. The observed resistance was strongly correlated with the Southern Runner variety's ability to maintain high kernel moisture under severe and prolonged drought. This trait was also inherited from the Brazilian parent. It was speculated that maintaining high kernel moisture kept the natural phytoalexin-based resistance functional longer during drought. Conventional breeding for drought tolerant peanut germplasm may be an effective approach to selecting for aflatoxin-resistant germplasm as well.

BREEDING FOR RESISTANCE TO AFLATOXIN CONTAMINATION IN ALMOND

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ABSTRACT

Previously identified seed coat barriers to infection by Aspergillus flavus Link were found to be relatively durable as well as stable over two growing seasons and at several central California locations, for almond cultivars, breeding lines, related species, and species hybrids tested. Fungal infection of the almond (Prunus dulcis [Mill.] D.A. Webb) kernel was observed following wounding of the seed coat or after prolonged exposures to high humidity. All cases of field infection were associated with seed coat and kernel damage, primarily by the navel orangeworm (Amyelois transitella [Walker]). Field levels of navel orangeworm infestation were found to be directly related to shell integrity. The very thick shelled European-type nut demonstrated a high level of resistance to both navel orangeworm infestation and A. flavus infection but resulted in low kernel-to-nut crack-out ratios. The basic components of the thick shell structure were a low density fibrous core between highly lignified inner and outer endocarp walls. A critical determinant of the degree of shell seal was found to be the integrity of the inner endocarp wall, particularly at the carpel suture union. Genotypes demonstrating a highly lignified inner endocarp wall but lacking the outer wall and much of the fibrous median tissue have been identified in species hybrids of almond with P. webbii and P. bucharica. Resulting nuts possess a strong, completely sealed endocarp, while achieving relatively high kernel-to-nut crack-out ratios of 0.5 to 0.6. Differences in navel orangeworm development rates on media supplemented with hull tissue from different genotypes were also observed.

Incorporation of these inner endocarp and outer mesocarp (hull) barriers to worm infestation, as well as seed coat barriers to fungal infection, into cultivars with good horticultural and yield performance is being pursued through controlled hybridizations between selected parents. The synthesis of vegetatively stable periclinal chimeras would allow a rapid manipulation of any or all of these barriers while leaving the horticulturally superior seed genotype intact. Opportunities for developing such 'histogenetically engineered' chimeras are now being explored using particle bombardment and micro-grafting approaches.

Gradziel, T.M. and Dechun Wang. (in-press). Susceptibility of California almond cultivars to aflatoxigenic Aspergillus flavus. HortScience

Gradziel, T.M. and D.E. Kester. (in-press). Breeding for resistance to aflatoxin contamination in almond. Acta Hort.

Summary of Panel Discussion: Can We Identify and Utilize Aflatoxin Resistance in Germplasm?

Panel Members: D. White, C. Holbrook, R. Cole, T. Wicklow, and T. Gradziel.

The discussion was began with general comments regarding disease resistance through conventional breeding. It must be understood that resistance is the ability of the host plant to suppress or retard disease and should not be confused with immunity, which is the complete absence of disease. Therefore, resistant varieties are those which will be less susceptible to *A. flavus* and produce less aflatoxin than those used currently. It is doubtful that we can completely eliminate aflatoxin, however, it appears that improved varieties used in conjunction with other control techniques can prevent aflatoxin formation to the extent that it will not cause marketing problems in most years.

The major requirement for identification and use of aflatoxin resistant germplasm is genetic variance for resistance. Work with peanuts, almonds, and corn has clearly demonstrated that substantial genetic variance is available in these hosts and that it is possible to identify high levels of resistance to both *A. flavus* and aflatoxin production. It is interesting that resistance to *A. flavus* and aflatoxin may be associated with other desirable traits. Resistance in almond is associated with resistance to navel orangeworm. Completely sealed shells confer resistance to navel orangeworms that are often responsible for creating wounds that are used for penetration by *A. flavus*. There may be an association of resistance to aflatoxin in peanuts and drought tolerance. It is not known if drought tolerant plants provide enough moisture to pods to prevent aflatoxin contamination under extreme drought conditions but drought tolerance may control aflatoxin in many environments. Research on corn has identified a number of different plant traits that are associated with resistance. It has been known for many years that tight husks and resistance to insect damage is related to resistance to *A. flavus*. More recently, traits such as chemical compounds in kernels and slow senence of silk has been implicated as being associated with disease resistance.

When enough diverse material is evaluated for resistance to aflatoxin production sources of resistance have been identified. Most of the sources of resistance have characteristics that are not agronomically acceptable and the levels for resistance must be bred into more agronomically acceptable varieties. This, however, does not seem to be an insurmountable problem.

There was some discussion on how quickly growers would begin to utilize aflatoxin resistant varieties. The concern is that aflatoxin resistant varieties may not be those varieties with the highest yield or best quality for marketable traits. This, however, can only be solved by time and effort necessary to incorporate the genes for resistance into the very best varieties.

At previous meetings a major concern was reliable and efficient screening techniques that could be used for identification of resistant plants. This was not discussed at length this year and it appears that we have identified methodology for screening plants. This may include creating conditions conducive for natural infection by *A. flavus* and aflatoxin production, testing of materials in locations where the problem exists, and artificial inoculation. All of these have advantages and disadvantages, however, the researchers seem to be able to deal with evaluation techniques. In addition, there was little discussion of the error variance of aflatoxin data. We all seem to accept the high c.v.'s that are associated with measuring a trait such as aflatoxin. In general, the problem of high c.v.'s is not ignored but is overcome by repeating the experiments in different environments which may be locations or years.

In summary there was no doubt that we have been able to identify sources of resistance and that genes for resistance can be incorporated into varieties with agronomically acceptable traits. Aflatoxin resistant varieties will achieve much wider use when their value is recognized by growers.

PLATFORM PRESENTATIONS

Other

Testing Commodities for Aflatoxin in the International Market

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USDA/ARS

SUMMARY

International trading of commodities susceptible to aflatoxin contamination puts a burden on both the exporter and the importer to determine if lot concentrations are above national aflatoxin guidelines. Lots rejected at the port of entry by the importer can result in large economic losses to the exporter. Exporters will test lots prior to shipment to reduce the chances of lots being rejected by the importer. The number of lots rejected by the importer depends to a large part on the differences between the exporter's and importer's sampling plans and sample acceptance limits.

Presently, the U. S. uses larger samples (three 21.8 kg) and higher sample acceptance limits (15 ng/g total aflatoxin) when testing peanuts for aflatoxin than its major trading partners. For example, the Dutch Code of Practice, depending upon the kernel count per ounce, uses either four 5 kg or four 10 kg samples and a sample acceptance limit of 3 ng/g B1 (about 5 ng/g total). The U.S. can reduce the number of lots rejected by the importer by reducing the sample acceptance limits to levels equal to or below that of the trading partner. Since 1988, the USDA has lowered the final sample acceptance level for the peanut aflatoxin sampling plan from 25 to 15 ng/g. It is estimated that decreasing the sample acceptance level from 25 to 15 ng/g has increased the number of lots rejected in the domestic market by

102 percent, has decreased the average amount of aflatoxin among domestic lots by 24 percent, and has decreased the number of lots rejected in the international market by 16 percent.

Present efforts by three CODEX Committees (Part of the Food and Agriculture Organization of the United Nations) to harmonize guidelines and sampling plans among member nations for peanut and corn products could remove differences between export and import sampling plans. FAO organized an Expert Consultation to design and evaluate 35 different sampling plans for peanut and corn products. The sampling plans represent two sample sizes (ranging from 3 to 27 kg) and five guidelines (ranging from 5 to 30 ng/g) for each product. Hopefully these plans will assist member nations in their deliberation to develop a common sampling plan and guideline for peanut and corn products traded in the international market.

THE SIGNIFICANCE OF STERIGMATOCYSTIN

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The purpose of this talk is to urge a slight expansion of the goals of the Multicrop Residue Program and related research programs for the elimination of aflatoxin from the food supply. The modification being sought is to add the goal of elimination of sterigmatocystin from the food supply.

Sterigmatocystin is a precursor of aflatoxin in those species that produce aflatoxins and is biosynthesized by a number of related fungi. Sterigmatocystin is an animal carcinogen and was listed , concurrently with aflatoxin, as a probable human carcinogen April 1, 1988 under the California Safe Drinking water and Toxic Enforcement Act of 1986 (Prop. 65). Under the Act, a no effect level of 0.02 μg/d was listed in January 1993. This is the same level as is set for aflatoxin. Under Proposition 65, a food manufacture has essentially three options when the Act is in force. The law can be ignored, product can be labeled, or the manufacturer can take the necessary steps to assure products are in compliance. No responsible company will ignore the Act or willingly label food products as potentially carcinogenic. This leaves only one course of action, that is, to take all necessary steps to assure that products are in compliance.

Manufacturers must not only be ready to defend against any regulatory actions or litigation but must also be ready to defend their actions in the court of public opinion.

It would serve little purpose to strive mighty to eliminate aflatoxin from the food supply and not eliminate the threat from sterigmatocystin, as far as, possible at the same time. Most of the research approaches such as resistance to fungi, insects, improved crop management, etc. offer the potential to reduce the incidence of sterigmatocystin as well as aflatoxin. Strategies to eliminate both mycotoxins would eliminate the need for the large amount of mycotoxin analysis and the expense of self regulation and legal compliance. It would also eliminate the potential need for warning labels on foods and reduce real and perceived risk to consumers. Because the research methods for elimination of aflatoxin seem to be the same ones that would be successful against sterigmatocystin, there seems to be no great need for additional research. There may, however, be some need for improvement in the methods to detect and quantitate this mycotoxin and to investigate resistance to a wider range of aspergilli and to include a wider range of aspergilli in ecological and other investigations of mycotoxin contamination of peanuts, corn, cotton seed, tree nuts and other commodities.

In summary, I urge you to broaden your research efforts where appropriate to include elimination of sterigmatocystin as an additional goal.

POSTERS

Pistachios - Characteristics Associated with Aflatoxin Formation

X-RAY IMAGE ANALYSIS OF INFESTED PISTACHIO NUTS FOR AFLATOXIN REDUCTION.

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INTRODUCTION: Sommer (Phytopathology 76:692, 1986) has reported that pistachio nuts harvested with intact hulls are essentially free of aflatoxin contamination. Aflatoxin was only found in pistachios where the hull was damaged allowing access to the mold spores. Among damaged nuts, insect infested nuts were two to three times as likely to contain aflatoxin. With present processing methods insect infested nuts are only removed if they show some other defect or if external evidence of infestation can be seen during the hand sorting operation. X-ray radiographs of pistachio nuts reveal internal evidence of infestation which is readily recognized by an experienced observer. Linescan x-ray conveyor belt systems are currently used to inspect baggage at airports. Similar systems with higher resolution sensors might be used to inspect food products.

OBJECTIVE: Develop machine recognition algorithms which could be used with x-ray linescan systems to remove infested pistachio nuts.

SAMPLES: Hulled, dried and sorted pistachio nuts were obtained from a commercial processor. In the commercial sorting process hollow shells, closed nuts, and stained nuts are rejected before sizing and final human inspection. Good nuts were sorted by size into four categories; fancy (20 nuts/oz.), export (21-22 nuts/oz.), domestic (22-23 nuts/oz.) and small (26-30 nuts/oz.). Naval orange worm infested nuts were obtained from the rejects of the final hand sorting operation.

IMAGE ACQUISITION METHOD: Due to the lack of x-ray linescan sensors of sufficient resolution in our laboratory, images were prototyped using x-ray film and a digital camera. Pistachio nuts were arranged on clear contact paper and radiographed at 25kvp for 2 min using Kodak Industrex B film in a Faxitron Series X-ray System 4380N. The resulting film was imaged at 0.083mm/pixel using a Videk Megaplus digital camera and the image transferred to a Sun workstation. The images were further reduced by pixel averaging to simulate resolutions of 0.25, 0.5 and 1.0 mm/pixel.

IMAGE ANALYSIS: Infested nuts generally appeared smaller and less bright than noninfested nuts. Therefore intensity histograms, normalized for area, were computed for each individual nut and category. The number of histogram bins was reduced from 256 to 16 with little loss of critical information. Fig. 1 shows the average histograms for each category at a pixel size of 0.25 mm. Note the convex shape of the right half of each noninfested histogram compared to the concave shape of the infested histogram. The bins showing the greatest differences between infested and noninfested nuts were grouped into light (pixel intensity 112 to 159) and dark categories (pixel intensity 16 to 47).

RESULTS: A plot of light versus dark is shown for each nut in figure 2. An area has been drawn which includes all the infested nuts and only two higher value nuts from the domestic and export grades. Six nuts with other defects and 5 small nuts are included in the rejection area.

DISCUSSION: These results suggest the potential for machine inspection and recognition of infested pistachio nuts which are more likely to contain aflatoxin than uninfested nuts. A separation surface has been drawn which separates infested nuts from high quality fancy, export, and domestic grades. The inclusion of the small nuts and nuts with other defects is not

serious because the nuts with other defects were being discarded with the infested nuts and small nuts are of lower value. Because of the limited number of images, these results must be considered preliminary and work is in progress to acquire a larger image data set, refine the image processing, and apply the work to real-time x-ray linescan images.

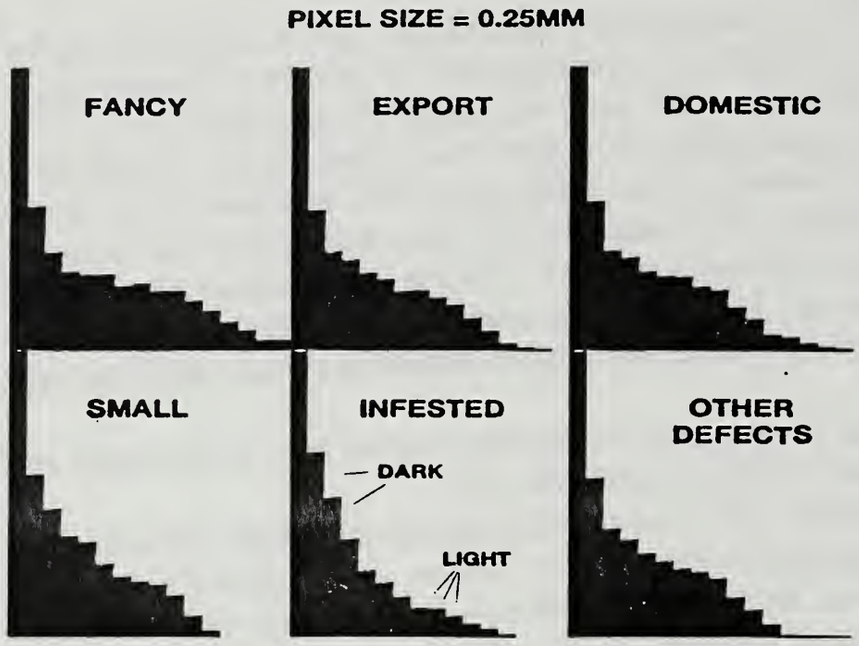

Fig. 1: Cumulative histograms for each nut category at a pixel size of 0.25 mm. The bins showing the greatest differences between infested and noninfested nuts were grouped into dark and light categories for subsequest analysis.

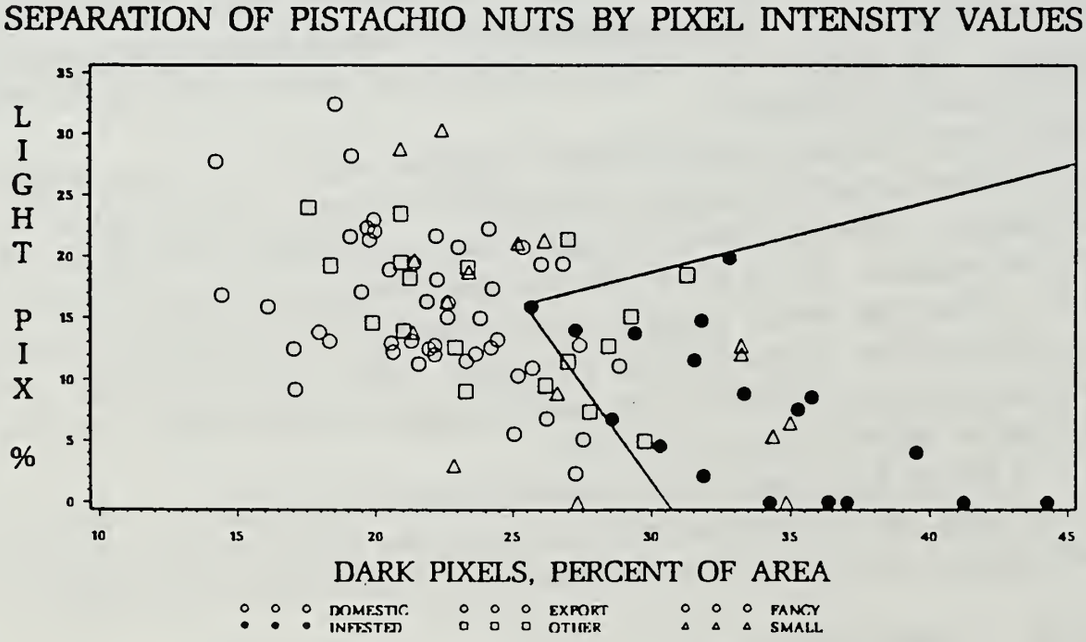

Fig. 2: Scatterplot of light (pixel intensity 112 to 159) and dark (pixel intensity 16 to 47) histogram bins for individual pistachio nuts. The line separates infested nuts from good product.

Characteristics of Pistachio Nuts with *Aspergillus flavus/parasiticus* and Aflatoxins

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Early splits (ES) are atypical pistachio nuts that have a split hull, exposing the kernel to invasion by molds and insects (normal nuts have intact hulls). *Aspergillus flavus* and *A. parasiticus* were found in 0.7 and 0.1% of the kernels of ES, respectively. Aflatoxins were detected in ES from six out of nine pistachio orchards in 1991 and five out of eight orchards in 1992. ES with rough, shriveled hulls had more than three times as much *A. flavus/A. parasiticus* infection as ES with smooth hulls. The rough ES had over 99% of the aflatoxins present in the pistachio nuts. The rough ES tended to have their hulls split earlier in the season than smooth ES with the result that the kernels of rough ES were exposed to colonization by molds for a longer period. Kernels infested by the insect navel orangeworm (NOW) (*Amyelois transitella*) had substantially more infections by *A. flavus/A. parasiticus* and 84% of all aflatoxins present. The combination of rough ES and NOW infestation had the most aflatoxin, although noninfested rough ES still frequently were contaminated with aflatoxins (Table 1). No aflatoxin was detected in smooth ES not infested with NOW and only a small amount in smooth ES infested with NOW. In addition to the detection of aflatoxins in pistachio kernels, the hulls of ES frequently had low levels of aflatoxins. Two other types of hull rupture besides ES, bird-damaged fruits and fruits with cracked hulls, had kernels infected with *A. flavus/A. parasiticus*, but at low levels. Rough ES had more NOW infestation, less weight, more shell discoloration, and smaller size than smooth ES, nuts with cracked hulls, or normal nuts with intact hulls. Fortunately, the pistachio nuts most likely to have mold and aflatoxin contamination (rough ES infested with NOW) had several physical characteristics (weight, size, shell discoloration, hull appearance) distinct from normal nuts that could facilitate removal during processing.

TABLE 1. The association of hull appearance and navel orangeworm (NOW) infestation with aflatoxins in kernels of early split pistachio fruits from three commercial orchards.

Characteristics of fruit	Number of 50-nut samples	Samples positive for aflatoxins (%)[a]	Aflatoxins (ng) per nut[b]	Percentage of total aflatoxins
Rough hull; NOW	18	61a	2998a	83.7
Rough hull; no NOW	51	20b	141b	16.2
Smooth hull; NOW	5	20abc	2c	0.1
Smooth hull; no NOW	47	0c	0c	0.0

[a] Numbers followed by the same letter are not significantly different (P=0.05) by pairwise comparisons using Fisher's exact test. No aflatoxins were detected in samples with intact hulls.

[b] The statistical analysis was performed on log transformed data. Numbers followed by the same letter are not significantly different (P=0.05) by pairwise comparisons using Fisher's LSD test.

SEPARATING "EARLY SPLIT" FROM NORMAL PISTACHIO NUTS FOR REMOVAL OF NUTS CONTAMINATED ON THE TREE WITH AFLATOXIN

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The hull of an early split pistachio nut abnormally splits open on the suture approximately 30 to 60 days before harvest. These nuts have be shown to contain nearly all of the aflatoxin in pistachio orchards[1]. Presumably, *Aspergillus flavus* molds contaminate early split nuts because of the easy access to the kernel through the hull opening. Early split pistachio nuts comprise of 1% to 4% of the crop so removal of these nuts to rid the pistachio crop from aflatoxin could be an economically sound method.

The physical properties of early split and normal pistachio nuts have been evaluated for possible implementation of a sorting criteria to remove early split nuts from the crop. The physical property investigation showed that early split nuts are significantly smaller in length, width, height, mass and volume than normal nuts. However, discriminate analysis (SAS, 1989) showed that these properties, used alone or in combinations, cannot be used to achieve a high sorting accuracy. However, these properties may be useful to enhance sorting accuracy with other methods. Unhulled nut moisture content, density, hull thickness, terminal velocity, and hull friction factor are not significantly different properties between early split and normal nuts.

Early split nuts tend to have more shell staining than normal nuts. Early split nuts are particularly prone to have either a yellowish hue stain all over the shell or they will have a dark brown stain adjacent to the suture split on the shell. Shell color was measured on a 2 mm diameter spot at the apex of the suture split on pistachio nuts. This spot often, but not always, contains a portion of the dark brown suture stain characteristic of early split nuts. Discriminate analysis (SAS. 1989) performed on the shell color data revealed that approximately 82% of the early split nuts could be removed from normal nuts while only 7% of the normal nuts would be erroneously removed. Nuts were also inspected for stains on the shell suture split or a yellowish hue covering at least 90% of the shell. When this categorical data was combined with the length and width of the pistachio shell, it was found that this data correctly classifies 90% of the early split nuts and 93% of the normal nuts.

Early split nuts have a strong tendency for their hulls to stick to their shells. Two devices were constructed to test the hulling characteristics of pistachio nuts. One device, called the spin huller, operated similar to a small potato peeler. It consisted of an abrasive rotating disk on the horizontal plane and enclosed with a vertical pipe lined on the inside with sandpaper. The other device tumbled the nuts at slow speed in a large drum. After 20 seconds in the spin huller, 98% of the normal nuts became hulled while only 7% of the early split nuts lost their hulls. After 180 seconds in the tumbler device, 99% of the normal nuts became hulled while only 9% of the early split nuts lost their hulls. It appears that hulling characteristics of nuts can be used to separate early split nuts from the crop without drastically changing current pistachio processing practices. Nuts that do not hull would be separated in color sorters or from a floating treatment.

An investigation to use computer vision to detect early split lesions on the hull of pistachio nuts was performed. Gray scale intensity profiles were computed across the width of the nut (perpendicular to the suture). If the profile crossed an early split lesion, there would be a deep and narrow valley on the profile at the early split location. Profiles were computed for every 0.5 mm on the nut and the number of adjacent profiles with deep and narrow valleys was recorded. Early split nuts would have a significantly higher count of adjacent profiles than normal nuts. Combining the length and width to the adjacent profile data, discriminate analysis (SAS, 1989), showed that 100% of the early split nuts and 99% of the normal nuts could be correctly classified.

[1]Sommer, N.F., J.R. Buchanan and R.J. Fortlage. 1986. "Relation of early splitting and tattering of pistachio nuts to aflatoxin in the orchard." *Phytopathology* 76: 692-694.

Treatments Which Eliminate the Observed Variability in Aflatoxin Production in Pistachios

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In the theory of preharvest aflatoxin formation we presented last year at this workshop, we discussed the phenomenon of variability. That is, of similar fresh nuts with similar *A. flavus* infections, one nut could contain 28 to 87,000 ppb AFB_1, and another could contain no aflatoxin. In this study fresh, autoclaved, dried/rehydrated and frozen/thawed pistachios were inoculated with small numbers (7, 100 or 200) of *A. flavus* spores. After four days at 25°C, aflatoxin was detected in the following percentage of fresh, inoculated nuts which had developed an *A. flavus* infection: 17% of dehulled nuts, 58% of "cut hull" nuts and 100% of early splits. Aflatoxin was detected in 100% of 38 autoclaved, dried and frozen nuts which had been inoculated. In autoclaved nuts, variable aflatoxin content appeared to be a function of water activity. Those fresh nuts which had been frozen and thawed prior to inoculation had the lowest average aflatoxin content. The "autoclaved + 40 % water" nuts had nearly three times the average aflatoxin content of the frozen nuts but were least variable in aflatoxin content. These data suggest that post-harvest treatment of pistachios affects cellular functions which can suppress aflatoxin production by *A. flavus*.

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POSTERS

Crop Handling and Storage

EFFECT OF MODULAR STORAGE ON THE INCIDENCE OF *ASPERGILLUS FLAVUS* CONTAMINATION OF SEED COTTON IN MISSISSIPPI IN 1992: A PRELIMINARY REPORT

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Aspergillus flavus group fungi were detected in seed cotton rinsate from 22.6% of the 31 crop sites sampled. Superficial populations found in this study were relatively low. Populations at three of the sites were less than 100 propagules/g, between 100-150 propagules/g at two sites, and two over 500 propagules/g of seed cotton. *A. flavus* was not detected in acid-delinted seed from any of the above crop sites but was detected from 2% of seed assayed from each of three other sites. *Fusarium* spp. were detected on lint from most sites and from 71% of the acid-delinted seed assayed for *Aspergillus*. *Fusarium* species most commonly encountered were *F. equiseti* (Corda) Sacc. and *F. semitectum* Berk. & Ravenel. Their rapid and luxuriant growth may have masked the detection of *Aspergillus* in this study. A cotton crop at Sidon, MS was selected for further study.

Module sampling ports (MSPs) were devised to permit repeated access to the interior of the module to obtain seed cotton samples and provide support for temperature sensors. An MSP consisted of a 10 cm dia. polyvinylchloride (PVC) pipe 61 cm long fitted with a cap on one end and a temperature sensor at the other. Seed cotton from the site was packed into the length of the MSP within a nylon hose to provide insulation from the external environment and to facilitate quick access for sampling. Four MSPs were spaced uniformly, with caps oriented outward, along each of the long sides of the module after two baskets, and again after five baskets, of seed cotton had been compacted. Immediately after module construction and after one week of storage, samples (ca. 0.5 lb) were pulled from the interior of the module via each MSP and assayed for *A. flavus* propagules.

A. flavus was recovered from three of eight MSPs within the Sidon module at the time the module was built and from 15 of 16 MSPs after one week of storage. Fungus populations sampled over time, (MSPs 1-8) increased in all but one case with storage. Populations generally were low except at one site where the seed cotton was noticeably damp when sampled. Moisture has long been associated with infection of cotton by *A. flavus*. Variation in temperature measurements among sampling sites was 3 F at moduling and, with the exception of one site, only 9 F after one week of storage. The errant and highest temperature was obtained at the site of the damp cotton previously mentioned and indicates increased microbial activity. Temperature in all cases was within the range suitable for growth of *A. flavus* and aflatoxin production. Assay of acid-delinted seed for aflatoxin indicated relatively small amounts from five of the 14 MSPs. The highest amount of aflatoxin detected was 14.4 ppb. Sufficient seed for analysis were not available from two MSPs.

This preliminary study collaborates reports of the existence of superficial populations of *A. flavus* in the cotton crop in the Mississippi Delta Region, describes a procedure for repeated access to a module for sampling over time, reports increases in *A. flavus* contamination of seed cotton with modular storage, and the presence of aflatoxin in seed samples after modular storage. This information provides a basis for continued study of the influence of modular storage of cotton on *A. flavus* infection and aflatoxin contamination of cottonseed. Two modules are currently under study and three more are contracted for study.

Acknowledgements

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POSTERS

Plant Resistance

Mechanisms of Resistance to Aflatoxin Production in Post-harvest corn

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Thirteen corn hybrids and one inbred were screened in the laboratory for susceptibility to aflatoxin production. Intact kernels and kernels wounded in the endosperm were inoculated by immersion in a suspension (10^6/ml) of *A. flavus* conidia and incubated 7 days. Based on patterns of aflatoxin production, corn genotypes were separated into three groups. Group 1 was characterized by very high aflatoxin levels in both wounded and intact kernels. Genotypes in Group 2 supported lower levels of aflatoxin production in intact kernels compared to wounded kernels. Group 3, containing only the inbred MAS: gk, evidenced drastic reductions in aflatoxin production regardless of wound. These results suggest that two mechanisms may function to limiting toxin production in corn kernels, i.e., structural factor(s) associated with intact kernels (Groups 2 & 3) and biochemical factor(s) (Group 3).

Two corn genotypes, which supported low (MAS:gk) and high (Pioneer 3154) levels of aflatoxin production, were used to study the role of the intact, cutinized pericarp in resistance to aflatoxin production. Treatment of intact kernels with 0.1 M KOH for 30 min. to extract cutin from the pericarp resulted in increased aflatoxin B_1 in MAS:gk to levels comparable to those in a susceptible hybrid. This treatment also had a similar effect on production of aflatoxin in Pioneer 3154. Adding diisopropyl fluorophosphate (DFP), a specific inhibitor of fungal esterase (cutinase), to the spore suspension reduced aflatoxin production in wounded kernels of MAS:gk and in kernels of Pioneer 3154. This suggests a role for cutinase in pathogenesis by *A. flavus*. Removing wax from intact corn kernels using hot chloroform resulted in increased aflatoxin levels compared with water treated or wounded kernels. Assays *in vitro* showed that *A. flavus* can grow using purified cutin as the sole carbon source and that it secretes extracellular cutinase, as evidenced by a yellow halo around the fungal colony after the plate was overlaid with the esterase substrate, *p*-nitrophenyl butyrate in agarose. These lines of primary evidence suggest that the pericarp on intact kernels plays an important role to reduce fungal growth and toxin production by *A. flavus*.

Effect of the Embryo on Aflatoxin Production in Excised Corn Kernels Inoculated with *Aspergillus flavus* in the Laboratory

N. Zummo and G.E. Scott

Four undamaged uninoculated ears from each of five corn genotypes were shelled and the kernels divided into four samples. The embryos were hand dissected from one sample from each ear and retained. The apical ends (approximately 2 mm) of the kernels in one sample were clipped and retained. The kernels in the remaining sample were left intact. Each of the three samples in individual Petri plates was sprayed with 1.7 ml of a conidial suspension (90,000,000 conidia/ml) of *Aspergillus flavus*. The plates were incubated in plastic boxes in a humid atmosphere at 28 C for 7 days when aflatoxin contamination from each sample was determined. The dissected embryos and clipped ends from each genotype were bulked, inoculated, and incubated at 28 C in individual Petri plates for 7 days. There was less than one ppb of aflatoxin in the uninoculated intact kernels of all genotypes. There was an average of 2,456 ppb of aflatoxin in the whole inoculated kernels, 128,800 ppb in the kernels with cut ends, and 62,700 ppb in the kernels with the embryos removed. In another test with Pioneer Brand 3165, there was 38,800 ppb of aflatoxin in kernels with cut ends and 54,100 ppb in kernels with embryos removed. It appears that an intact pericarp in corn kernels influences aflatoxin contamination as much or more than presence or absence of the embryo.

Infection of Corn Silks by *Aspergillus flavus*

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The silk channel is the obvious avenue of entry for *Aspergillus flavus* or other kernel-infecting fungi into undamaged corn ears. Attempts to infect corn kernels with *A. flavus* utilizing silk inoculation techniques have yielded erratic results. Those inoculation techniques that have been most successful have been those that injured the ears and/or kernels with the exception of Zummo and Scott who applied their inoculum directly over the kernels through the silk channel or through the husks. Failure to infect corn kernels with *A. flavus* using silk inoculation techniques prompted us to investigate the role of corn silks in the infection process. Silks on some genotypes remained live, healthy, and intact for up to 20 days after emergence. Some silks, underneath and protected by the husks, remained intact for up to 30 days after emergence. We did not separate genotypes based on silk longevity. When individual non-surface, sterilized early silks were plated on CSA-S, which permits the growth of *A. flavus/parasiticus* but inhibits the growth of most other microbes, there was no fungal growth on the inact portions of the silks or on the agar adjacent to the silks after 5 days. There was fungal growth only at the cut ends of the silks or in areas where the silks had been broken. There was copious fungal growth from individual late silks plated on CSA-S. When 3-inch pieces of early silks, intact midsilks, and late silks, were inoculated with three *A. flavus* isolates or one *A. parasiticus* isolate, NRRL 2999, using three inoculation techniques in the laboratory and incubated for 5 days, there was little or no fungal growth on the early silks. The growth on the intact midsilks was restricted at the ends and at broken places on the silks. There was copious growth and sporulation on the brown silks and adjacent medium. Corn silks, as they emerge from the ear, have some apparent resistance to infection by *A. flavus/parasiticus*. As the silks senesce, the resistance is lost and the fungus is able to invade the dead or moribund silks. Our results may be at variance with the results of other researchers, but these findings may help to explain some of the erratic results from silk inoculations in earlier work. We did not explore the presence of other microorganisms that may be antagonistic or inhibitory to growth of *A. flavus/parasiticus* on corn silks.

Aflatoxin Elimination Workshop
Little Rock, Arkansas, October 24-26, 1993

EVIDENCE IMPLICATING THE LIPOXYGENASE PATHWAY FOR PROVIDING RESISTANCE TO SOYBEANS AGAINST *ASPERGILLUS FLAVUS*

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The fungus, *Aspergillus flavus* secretes lipases while parasitizing oil-seed hosts and may contaminate the seed with aflatoxin. We have found that exogenous lipase applied to soybean cotyledons results in the generation of volatile aldehydes by the lipoxygenase pathway that halts the growth of fungi. Volatiles generated from untreated homogenized soybeans did not affect the germination of *A. flavus* spores in a closed Petri dish assay, in which spores were streaked on corn meal agar in the bottom half of the Petri dish and soybean homogenate was placed inside the lid of the inverted plate. When lipase (500 units/ml) was added to the soybean homogenate, all fungal spore germination was inhibited. However, the addition of nordihydroguaiaretic acid (a lipoxygenase inhibitor) to the soybean homogenate/lipase mixture partially reversed the inhibition of spore germination. The extent of spore germination was dependent on both the number of soybean cotyledons in the homogenate, and the activity of the added lipase. Addition of linoleic or linolenic acids to the homogenates also inhibited spore germination, whereas palmitic, stearic, or oleic acids had no effect, compared to untreated homogenates. Headspace analysis indicated that hexanal, a product of the lipoxygenase pathway with known antifungal activity, was the major volatile generated from lipase-treated homogenates. Soybeans are exceptional among oil-rich seed crops in that they are resistant to *A. flavus* infection and aflatoxin contamination. Our evidence strongly suggests that the lipoxygenase pathway may contribute to this resistance.

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POSTERS

Microbial Ecology and Control Techniques

SAP BEETLE IPM FOR MYCOTOXIN CONTROL IN CORN: 1993 RESULTS

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Sap beetles are important vectors of mycotoxigenic fungi to corn. A multicomponent IPM program with potential for area-wide management of mycotoxins in corn is being developed with emphasis on the dusky sap beetle, the most widespread and destructive sap beetle in corn in the United States.

Two releasers for ethyl acetate and propanal attractants typically lasted 3 weeks and varied less than 10% in release rate. The ethanol releaser was more variable due to inconsistent materials. When all were optimized, attractancy was equivalent to the short-lived fermenting bread dough.

The mermithid nematode parasite was only found at 1 of 19 sites in Central Illinois, but infection at this site was at least 50% in early spring. *Beauveria bassiana* was widely distributed at these sites, but killed 3% of sap beetles at most.

A corn hybrid with previously demonstrated resistance to *A. flavus* sustained only 2% damaged kernels (range 0 to 26%) in off-ear assays, and prevented invasion of 50% of silk-infested corn earworms (range 7 to 55%). A *F. moniliforme*-resistant hybrid sustained only 3.4% damage in off-ear kernel assays and prevented invasion of 48% of corn earworms. When husks were removed, ears were damaged by sap beetles, birds, and corn rootworm adults; but some hybrids still sustained relatively less damage. Differential odor-based attractancy may be involved, as well as differential access to kernels by open husk tips, silk channel fill, husk tightness, and kernel blockiness.

Additional "peroxidases" were present/more active in varieties with brown pericarp compared to less brown ones, and ferulic acid (a common pericarp component) was oxidized 2- to 3-fold more rapidly. Corn powder containing ferulic or courmaric acid that was enzymatically browned increased mortality of corn earworms compared to controls (from 36 to 75%) and deterred feeding by sap beetle adults by up to 1.8-fold.

Granular encapsulated malathion was avoided by lady beetles when aphid prey (67% mortality without aphids, 12.5% with) or water (62.5% mortality without water, 22.5% with) was present in laboratory assays. In contrast to effectiveness noted in 1992, treatments were less effective, due presumably to heavy and frequent rains. Spray malathion (5 applications) reduced sap beetles and corn earworms by 1.8-fold at milk stage and total insects by 1.3-fold at harvest. Granules reduced total insects by 1.5-fold at harvest. The multiple sprays and granules reduced *Fusarium* spp. at harvest by 1.4-fold and 1.6-fold, respectively. *Steinernema* nematodes, which can control corn earworms, killed 49% of driedfruit beetle larvae in laboratory assays.

When the fungal biocompetitor *Bacillus subtilis* was distributed to damaged corn ears by sap beetles in laboratory assays, *A. flavus* colonization was prevented by 41% when sap beetle access was allowed prior to inoculation with *A. flavus*, as indicated by visible moldiness. In field tests, 100% of ears that allowed access of sap beetles potentially picking up *B. subtilis* in autoinoculators prior to *A. flavus* inoculation were free of *A. flavus*, as indicated by visible moldiness, compared to 7% for ears that allowed access after *A. flavus* inoculation.

Distribution of Atoxigenic Strains within Populations of *Aspergillus flavus*.

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During cultivation, fungi grow on crops and crop associated debris. This results in fungi, including the Aspergilli, becoming associated with agricultural products. Application of select strains of *Aspergillus flavus* to soil or crops during the early stages of crop cultivation provides the opportunity to improve the safety of fungal populations associated with crops and agricultural products, and, at the same time, reduce the ability of populations to produce aflatoxins and contaminate crops. In field tests performed on cotton in the desert valleys of Arizona, certain atoxigenic strains (i.e. strains which do not produce aflatoxins) have been shown to reduce aflatoxin contamination when applied to the crop early in the season. Fungi inevitably become associated with crops during production. Application of select fungal strains to soils early in crop production provides the opportunity to select which fungi will be associated with crops rather than allowing chance to determine the safety of these fungal populations.

Control of aflatoxin contamination with atoxigenic strains in diverse locations and on diverse crops may require the use of diverse strains with a wide variety of various adaptive traits. Furthermore, selection of strains for optimal safety may require identification of strains with reduced expression of other traits such as production of other toxins, various enzymes, and resistance to antifungal agents. *Aspergillus flavus* populations are composed of many distinct genetic groups called Vegetative Compatibility Groups or VCGs. These VCGs are separately evolved lines of *A. flavus* with different traits. Strains within a VCG tend to produce similar levels of aflatoxins (see Bayman and Cotty, 1993, Can. J. Botany 71:23-31). Some VCGs produce no aflatoxins at all. VCGs also differ in competitive ability and many other traits. Variability among VCGs suggests the potential extent to which optimal strains may be selected.

In order to obtain a better understanding of populations of toxigenic and atoxigenic *A. flavus* strains, we examined the distribution of atoxigenic vegetative compatibility groups in several cotton-producing areas. We found that the distribution of VCGs differs among areas, suggesting that *A. flavus* strains may have specialized adaptations that permit differential success at various locales. This observation may be important in the selection of strains for control of aflatoxin contamination at various locations. Furthermore, certain VCGs occur much more frequently than others, suggesting that these VCGs are the more successful genotypes and more competitive.

Twenty percent of *A. flavus* isolates collected from 32 fields in five states failed to produce aflatoxins in liquid fermentation (limit of detection 1 PPB). Atoxigenicities of most VCGs were stable regardless of treatment. However, isolates in a few VCGs produced aflatoxins when either mutated into a *niaD*$^-$ phenotype or transferred by single conidium. Stability of atoxigenicity may be an important trait for selection of atoxigenic strains for use as agents for preventing contamination. The 199 isolates initially identified as atoxigenic belonged to 59 vegetative compatibility groups. Fifty-two percent of the atoxigenic strains examined belonged to one of nine VCGs. Many VCGs were represented by only single isolates. These studies indicate a great diversity of genotypes from which to select strains for use as agents to prevent aflatoxin contamination.

Bacterial Chitinases for the Control of Aflatoxin Contamination

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Chitinases are antifungal enzymes which degrade fungal cell walls by hydrolyzing β-1,4-linkages of chitin present in many plant pathogenic fungi. In our previous studies we identified a chitinolytic bacteria, Bacillus chitinosporous S (AU192) which inhibited Aspergillus flavus, A. parasiticus, and Fusarium moniliforme. Tritiated chitin was used as substrate to determine the chitinase activity of the culture filtrate and crude protein extract of AU192. A gel overlay technique utilizing glycol chitin as substrate was used to detemine number of chitinases in the crude protein extract. Results show that AU192 produces at least 3 chitinases. The antifungal activity of the culture filtrate against A. flavus was tested by the microtitre plate reader technique. Boiling the culture filtrate significantly reduced inhibition. The culture filtrate fraction having proteins greater than 10 kD showed greater inhibition than fraction having proteins less than 10 kD. The boiled fractions showed less inhibition. These results indicate that the primary antifungal compounds are greater than 10 kD in size. Microscopic observation of the fungal mycelia in the wells containing culture filtrate showed hyphal tip bulging indicating chitinase activity. These results indicate that chitinases are at least partially responsible for the antifungal activity against A. flavus. Studies are underway to isolate chitinases from the culture filtrate of AU192 and test their antifungal activity. Additionally, studies to construct a genomic library of AU192 in E. coli, and B. subtilis in order to identify the genes encoding the most active chitinase are underway.

POSTERS

Chemical Defenses and Methodology

HPLC Method for the Analysis of Stilbene Phytoalexins

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Phytoalexins are low molecular weight, biologically active metabolites produced in plants as defense reactions in response to various exogenous stimuli.

Peanuts have been shown to produce stilbene type phytoalexins (SP) in response to fungal infection, wounding and UV-irradiation. SP have been demonstrated to inhibit fungal growth and spore germination of *Aspergillus* and *Penicillium* species. SP appear to be partly involved in the resistance to drought-induced, preharvest aflatoxin contamination of peanuts since: (1) SP are naturally produced in field-damaged peanuts; (2) they possess biological activity against *A. flavus* and *A. parasiticus*; (3) although invasion of peanuts by *A. flavus* and *A. parasiticus* can occur under any conditions, aflatoxin contamination does not occur until peanuts lose the capacity for SP production as a result of drought-induced kernel dehydration.

Unfortunately, knowledge of the role of SP in peanuts is limited because of lack of an adequate analytical base; the existing chemical methods cannot provide needed reliability, high sensitivity, accuracy and simplicity.

The purpose of this work was to develop a simple, fast and accurate method of quantitation of SP in peanuts.

An HPLC method for the determination of (SP) in peanuts has been developed. SP were extracted with CH_3CN-H_2O (9:1, v/v) using a high-speed blender. An aliquot of the extract was applied to a minicolumn, packed with Al_2O_3-ODS(C_{18}) mixture and eluted with CH_3CN-H_2O (9:1). The eluate was evaporated by N_2, and the residue dissolved in HPLC mobile phase. SP in an aliquot of the purified extract were quantitated by means of normal-phase partition HPLC on Silica Gel using *n*-Heptane - 2-Propanol - Water - Acetonitrile - Acetic acid (1050+270+17+5+1, v/v) as mobile phase. Recoveries of SP [*t*-Arachidin-3 (*t*-Ar-3), *t*-3-isopentadienyl-4,3',5'-trihydroxy-stilbene (*t*-IPD) and *t*-Resveratrol (*t*-Res)] from peanuts, spiked over 200 ppb, were 96.05±2.8%. The limits of quantitation for the above SP were about 100 ppb. The method demonstrated good reliability and simplicity and was used to find that:

1. Peanuts invaded by fungi produced exclusively *trans*- isomers of SP.
2. All parts of peanut plant (leaves, stems, roots, kernels and pegs) were able to produce the same SP in response to fungal invasion.
3. In all our experiments peanuts produced *t*-Ar-3, *t*-IPD and *t*-Res as major SP in varying proportions.
4. SP were produced by peanuts in response to *A. flavus* and *A. parasiticus* as well as natural microflora invasion.
5. Different varieties of peanuts (both cultivars and wild species) demonstrated unequal ability to produce SP.

ISOLATION, CHARACTERIZATION AND BIOACTIVITY OF STEROL ESTERS OF CINNAMIC ACID DERIVATIVES FROM CORN BRAN

Aflatoxin Elimination Workshop--Little Rock, AR, October 24-26, 1993

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ABSTRACT

Esters of sterols and cinnamic acid derivatives (CADs) comprise a group of compounds found, among others, in seeds of the cereal grains corn, wheat, rye, triticale and rice. In order to obtain pure components for biological testing an improved method of separating these compounds was developed. Steryl CAD fractions were obtained by hexane extraction followed by silica gel chromatography using column and Chromatotron methods. An HPLC method using acetonitrile:n-butanol:acetic acid:water (94:3:2:1, v/v/v/v) gives improved resolution of steryl esters of CADs isolated from corn bran and indicates that this group of compounds is more complex than previously reported. Composition and retention times, relative to ergosterol and cholesteryl cinnamate, for a total of sixteen compounds which appear to be steryl CADs are reported. A simple method for hydrolysis and joint determination of the CAD and sterol portions of the ester is described. Exclusion of oxygen by flushing the hydrolysis mixture for 30 seconds with N_2 allowed nearly complete recovery of caffeic acid; however, no steryl esters of this compound or CADs other than ferulate and *p*-coumarate were found. Sitosteryl, sitostanyl, stigmasteryl, campesteryl, campestanyl and Δ^7-sitosteryl and Δ^7 campesteryl ferulates, have been identified in corn bran and MS data for the TMS derivatives of the sterols esterified to ferulic and *p*-coumaric acids are given. In addition to known *p*-coumarates campesterol and Δ^7-campestenol forms were found.

The complete hexane fraction which was the source of the steryl esters was separated by TLC into 7 fractions which were tested for activity on *Aspergillus flavus* NRRL #6536, a known aflatoxin producer. The incubation system used a defined liquid medium (SL) and vials with open caps and filter paper septa, allowing greater air exchange. Hexane extract and subfractions from corn bran tested at 5X the level occurring in bran had a dramatic effect on growth and aflatoxin level: the most active fractions for growth were a fraction containing a group of compounds intermediate in polarity between the steryl CADs and triglycerides. Two other franctions contained di-, mono-, and desmethyl sterols along with other unknown components; growth increased 5.6, 8.4 and 6.5 times the Tween-80 control respectively. The steryl CAD fraction from corn resulted in 2.3 times the growth of the control; rice CADs had no effect on growth but did allow synthesis of aflatoxin at 1/3 of the control. Tween-80, at the level used, resulted in about 65% inhibition in growth and 70% decrease in aflatoxin. There was essentially no aflatoxin synthesis with any of the TLC fractions or the complete extract; the reason for this is not clear.

Aflatoxin Elimination Workshop
Little Rock, Arkansas, October 24-26, 1993

CHEMICAL DEFENSES IN SURVIVAL OF *ASPERGILLUS* SCLEROTIA

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Our research program is organized with the goal of integrated pest management of *Aspergillus flavus* and aflatoxin. One of our objectives is to investigate factors limiting the survival or germination of *A. flavus* sclerotia in both irrigated and non-irrigated corn field soils. In addition to *A. flavus*, other sclerotium-forming aspergilli classified in the *Aspergillus ochraceus* group or *Aspergillus niger* group have been recorded from corn kernels, peanuts, cotton bolls and pistachio fruits. These aspergilli also colonize decomposing crop residues and may reach the developing fruit as air-borne inoculum or be simultaneously vectored to crops by nitidulid beetles or other pest insects. *Aspergillus niger* has been the subject of numerous investigations on its ability to interfere with *A. flavus* and aflatoxin, while members of the *A. ochraceus* group produce the mycotoxin, ochratoxin A. We are conducting general studies of the chemistry of *Aspergillus* sclerotia as sources of new antiinsectan natural products that protect these survival structures from soil-inhabiting fungus-feeding arthropods. Our research has shown that while each species of *Aspergillus* has a unique sclerotial chemical defense system (profile of defensive compounds), individual metabolites such as nominine may be common components of the defense systems of different species.

Investigations of antiinsectan *Aspergillus* sclerotial metabolites are in collaboration with P.F. Dowd, Mycotoxin Research Unit, NCAUR, Peoria, and J.B. Gloer, Department of Chemistry, Univ. of Iowa. The research is supported by a grant from the National Science Foundation and through a Cooperative Research and Development Agreement No. 58-5114-0-4003 with the Biotechnology Research and Development Corporation, Peoria, Illinois.

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Quantitative HPLC Analysis of Aflatoxins in Aspergillus Extracts using Aflatest™ Immunoaffinity Column

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Aflatoxins B1, B2, G1, and G2 are mycotoxins produced by some strains of Aspergillus *flavus* and almost all strains of Aspergillus *parasiticus*. A. *flavus* produces primarily aflatoxin B1 along with smaller amounts of B2; A. *parasiticus* produces primarily aflatoxin G1 along with smaller amounts of B1, G2, and B2. Accurate identification of the aflatoxins produced by Aspergillus cultures is an important consideration in chemotaxonomy and the biochemistry of the aflatoxins. Reliable quantitation of aflatoxins produced by cultures is important for the *in vitro* screening of chemical compounds for aflatoxin biosynthesis inhibition.

Thin-layer chromatography (TLC) has been the primary method used for the identification of aflatoxins by Aspergillus cultures. HPLC, in combination with the Aflatest™ immunoaffinity column, has many advantages over TLC in the qualitative and quantitative analysis of aflatoxins from Aspergillus cultures. The Aflatest™ column isolates only aflatoxins B1, B2, G1, and G2 from Aspergillus extracts allowing removal of extraneous fluorescent compounds which could interfere with aflatoxin identification. Aflatoxins B1 and G1 must be derivatized with trifluoroacetic acid to enhance their fluorescence in the polar solvents required for reverse-phase chromatography; aflatoxins B2 and G2 do not require derivatization. The detection limit for each aflatoxin by HPLC is 0.1ng versus 5ng for TLC. Recoveries of individual aflatoxins from the Aflatest™ are linear in the range 5-200ng, with 87%, 89%, 85%, and 70% recoveries for B1, B2, G1, and G2, respectively. Recoveries of The Aflatest™ shows greatest affinity from an aflatoxin matrix for aflatoxin B1, followed by B2, G1, and G2. Recovery of B1, B2, and G1 is linear up to 150ng each from a 600ng total aflatoxin matrix, and recovery is linear for aflatoxin G2 up to 100ng from a 400ng total aflatoxin matrix.

Visualization of Mycotoxin Production in Maize Tissues.

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Summary

Different *Aspergillus* species (*flavus*, *parasiticus* and *nidulans*) producing different intermediates and end-products of the aflatoxin pathway (norsolorinic acid, NOR; sterigmatocystin, ST and aflatoxin, AF) are useful in studying the maize/*Aspergillus*/mycotoxin interaction. *Aspergillus flavus* and *A. parasiticus* AF mutants which produce NOR, a visible orange intermediate of both ST and AF, were used to visualize mycotoxin deposition in host and fungal tissues. *Aspergillus nidulans*, a ST-producer, was found to follow a similar pattern of colonization and mycotoxin production in maize kernels as *A. flavus* and *A. parasiticus*. Results indicate that these mycotoxins are produced and accumulate in specific maize kernel tissues (embryo and aleurone) and specific fungal tissues (substrate mycelium and not sporulating mycelium). In field maize inoculated with *A. parasiticus*, NOR was visibly easier to detect than fungal mycelium. All kernels which showed visible NOR were heavily infested with *A. parasiticus*. NOR pigmented kernels were randomly scattered on the ear and it appeared from the pattern of NOR accumulation that the fungus typically gained access to the kernel at the embryo and then moved up through the aleurone tissues. We suggest that NOR mutants may be useful tools to identify likely infection sites in maize kernels and that the genetically characterized *A. nidulans* may be useful in helping identify global regulatory mechanisms in the maize/*Aspergillus*/mycotoxin interaction.

Introduction

Aspergillus spp. are seed deteriorating fungi known for their ability to produce mycotoxins in such crops as maize, peanuts, tree nuts and cottonseed. Aflatoxin (AF) and sterigmatocystin (ST), end-products of the same biosynthetic pathway, are two of the most common *Aspergillus* mycotoxins. AF is produced by *A. flavus* and *A. parasiticus* and ST is produced by several *Aspergillus* spp. including *A. nidulans* . A significant limitation in understanding the regulation of AF and ST biosynthesis in the host plant has been the lack of simple microbiological assay systems. We have developed informative microbiological assays by using different *Aspergillus* species which produce either norsolorinic acid (NOR), ST and/or AF. NOR is a highly visible orange intermediate in the AF/ST biosynthetic pathway consisting of: initial polyketide precursor > norsolorinic acid > averantin > averufanin > averufin > versiconal hemiacetal acetate > versicolorin A > sterigmatocystin > O-methylsterigmatocystin > AFB_1. In the present study, we used NOR-producers to see where and when mycotoxins are produced in intact maize

kernels and a ST-producer to identify differences or similarities in fungal colonization and concomitant polyketide mycotoxin production by *Aspergillus nidulans*. A more detailed report can be found elsewhere (2).

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Figures 1A-D. Living maize kernels infested by *Aspergillus spp.* A-C. Ungerminated seed of maize hybrid TX804 x TX814. All three *Aspergillus spp.* preferentially colonize the embryo of living, ungerminated maize kernels. *A. parasiticus* SK and *A. flavus* Papa 827 are white-spored isolates which produce both NOR (the reddish-color under the conidia) and AF (not visible). *A. nidulans* FGSC 26 produces dark spores and ST (not visible). **D.** Colonization of embryo inoculated maize seed after 1, 2, 3 and 4 days by *A. flavus* Papa 827 and *A. parasiticus* SK. When the seed is cut in half and spores washed off, the spread of NOR (red coloration) is seen in the embryo. Although NOR appears to remain largely within the fungal mycelium, it will spread to the endosperm with time as will AF and ST (Table 1).

Figure 2A-B. Accumulation of NOR in aleurone tissue of the maize kernel. A. When endosperm inoculated seed are dissected into endosperm, pericarp and aleurone tissues, NOR is found only in the aleurone tissue. **B.** A cyrostat section of an endosperm inoculated seed (courtesy of Dr. L. Lindner). Note that both fungal hyphae and NOR accumulation are largely restricted to the aleurone layer. Some hyphae appear fairly free of NOR whereas other hyphae contain concentrated amounts of NOR. Starch granules are visible below the aleurone layer.

Figure 3. Visualization of NOR in a field-inoculated ear of hybrid NC + 6414. Ears of hybrid NC + 6414 were inoculated at the milk stage with a spore suspension of *A. parasiticus* SK and then collected at harvest (courtesy of Dr. J. Dunlap). Although fungal mycelium was not always readily apparent, NOR was readily visible in the aleurone layer of several kernels (note red kernels in photograph). All of the kernels on this ear (excepting kernels damaged by the inoculation technique) were bissected and scored for visual presence of NOR in the embryo, endosperm and/or aleurone tissues (Table 2). Kernels were then placed in a humidity

chamber and observed on day 2 for presence of vigorous fungal growth. Typically in NOR pigmented tissues, the embryo would be entirely pigmented, the aleurone partially or entirely pigmented, and the endosperm partially pigmented. In those kernels where the endosperm was pigmented, NOR was located in the tissues next to the scutellum or aleurone. In most of the kernels where NOR was seen in the aleurone and/or endosperm but not in the embryo, the embryo had died and turned brown which would mask the presence of NOR. It was probable that there was NOR in the embryo of these kernels.

Table 1. Sterigmatocystin, norsolorinic acid and aflatoxin accumulation in parts per million in embryo-inoculated maize kernels.

	3 day[a]		6 day		9 day		12 day	
	Em[b]	En	Em	En	Em	En	Em	En
A. nidulans FGSC 26[c]								
ST	308.2[d]	0	336.6	11.1	0	0	360.8	0
A. parasiticus SK								
NOR	510.1	0	2307.2	6.4	417.0	6.3	710.6	0.3
AFB_1	14.6	0	75.7	0.3	88.4	3.2	49.1	0
AFB_2	7.6	0	22.3	0	27.7	0	0	0
AFG_1	18.3	0	387.6	2.5	336.2	19.7	310.2	1.1
AFG_2	3.1	0	51.0	0	45.4	2.1	43.3	0
Total AF	43.6	0	536.6	2.8	497.7	25.0	402.6	1.1
A. flavus Papa 827								
NOR	2.6	0	138.2	0.4	1.1	0	319.4	1.1
AFB_1	2.4	1.2	28.4	0.1	1.3	0.3	94.3	0.5
AFB_2	0	0	4.8	0	0	0	14.2	0
AFG_1	0	0	0	0	0	0	0	0
AFG_2	0	0	0	0	0	0	0	0
Total AF	2.4	1.2	33.2	0.1	1.3	0.3	108.5	0.5

[a] Maize kernels were wounded by piercing the pericarp covering the embryo with a sterile pin following Brown et. al. (1). Inoculum consisted of 5 µl of a spore suspension [10^6 spores/ml] placed on the wound. Treatements consisted of ten kernels incubated at 29°C in the dark for 3, 6, 9, or 12 days.

[b] Em = embryo; En = endosperm + aleurone. Ten kernels per treatment were separated into embryo and endosperm + aleurone tissues and these tissues were extracted separately for mycotoxins.

[c] *Aspergillus nidulans* FGSC 26 produces sterigmatocystin (ST); *A. parasiticus* SK produces norsolorinic acid (NOR), and the following aflatoxins (AF): AFB_1, AFB_2, AFG_1, AFG_2; *A. flavus* Papa 827 produces norsolorinc acid (NOR) and the following aflatoxins (AF): AFB_1 and AFB_2.

[d] Mycotoxins are reported in parts per million.

Table 2. Presence of norsolorinic acid (NOR) and *Aspergillus parasiticus* SK in 417 maize kernels from one ear.

Kernel Tissues[a]	# of Kernels[b]	% SK[c]
Em	31	100
En[d]	1	100
A[d]	18	100
Em/En	3	100
Em/A	37	100
En/A[d]	11	100
Em/En/A	15	100
No NOR	291	9.6
Total[e]	417	154

[a] Kernels were assessed as to whether NOR was visible in just the embryo (Em); just the endosperm (En); just the aleurone (A); embryo and endosperm (Em/En); etc. or if NOR was not visible at all (No NOR).

[b] The number of kernels in each of the described tissue groups (e.g. 31 out of 417 kernels had visible NOR only in the embryo).

[c] The percent of kernels in each of the described groups which were colonized with *A. parasiticus* SK two days after incubation in a moist chamber.

[d] In many instances where the endosperm and/or aleurone were contaminated with NOR, the embryo had died and turned dark brown thus masking the potential presence of NOR in this tissue. It is probable that many, if not the majority, of kernels in these categories had significant quantities of NOR in the embryo.

[e] 417 kernels were assessed for NOR contamination and fungal growth. 126 kernels were contaminated with NOR and 154 kernels (including the 126 NOR kernels) were colonized by *A. parasiticus* SK as described.

POSTERS

Molecular Biology

Construction of Plant Transformation Vectors for Expression of GUS and Antifungal Traits. J. W. Cary, T. E. Cleveland, and T. J. Jacks, USDA-ARS, SRRC, New Orleans, LA; C. A. Chlan and S. Kunning, University of Southwestern Louisiana, Lafayette, LA; and J. Jaynes, Demeter Biotechnologies Inc.

Current strategies for the control of fungal diseases in crops have focused on the use of plant genetic engineering to introduce resistance genes into susceptible plant cultivars. These resistance genes may encode enzymes which directly effect the growth of the fungi (i.e. chitinases, glucanases, and osmotins) or they may encode inhibitors of fungal enzymes required for successful invasion of the host plant (i.e. inhibitors of proteases, pectinases, and amylases). Acquired resistance to a fungal species often requires that these genes be isolated from sources other than the host plant. However analogous host genes may also be effective if placed under the control of promoters that express the gene in an optimal temporal and spatial manner.

We are interested in developing transgenic cotton varieties that demonstrate resistance to invasion by aflatoxigenic strains of *Aspergillus flavus* and/or reduced aflatoxin production. The success of this project will depend in large part upon the identification and isolation of a gene(s) that will provide the desired traits. To date , we have not identified any one gene product that is capable of conferring resistance to *A. flavus* or decrease aflatoxin production. However we have identified possible sources of fungal/aflatoxin inhibitory factors in our lab as well as obtained genes from other sources that have previously demonstrated antifungal activity. These genes will be used both individually and in concert with one another in the development of vector constructs for transfer into cotton via *Agrobacterium* or biolistic transformation techniques. Transgenic plants will then be analyzed for either reduction in aflatoxin levels or resistance to *A. flavus* and other common cotton pathogens.

In addition to generating vectors harboring antifungal/antitoxin genes, we will also be designing vector constructs that will express these genes at desired times (induced versus constitutive expression) or within the cottonseed versus whole plant expression. Initial work in this area has focused on expression in tobacco of the cotton seed storage protein gene (B gene) or a GUS reporter gene under the control of either the B gene promoter or a phaseolin promoter. The levels of resistance due to targeting of the gene products within the plant will also be studied. In many cases genes that normally encode sequences for vacuolar targeting of the product will be engineered so as to be transported to the intercellular spaces.

ABSTRACT

Comparative mapping of aflatoxin pathway gene clusters in *A. flavus* and *A. parasiticus*

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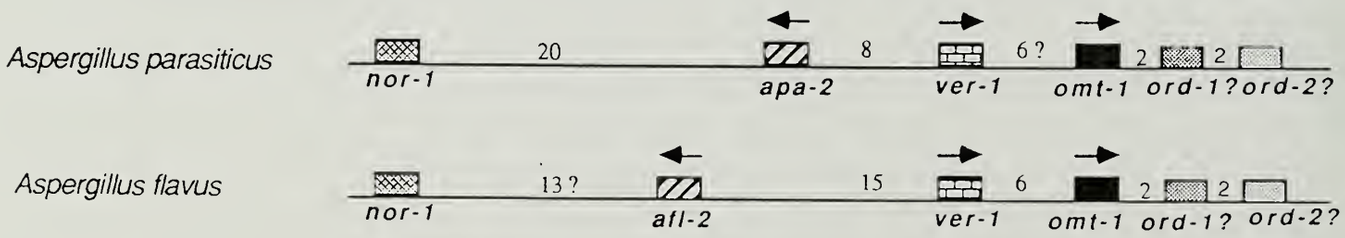
Aflatoxins are secondary metabolites produced by the fungi Aspergillus flavus and A. parasiticus. They are potent carcinogens to animals and are a potential health hazard to human beings. The aflatoxins are synthesized by condensation from acetate units. The biosynthesis of aflatoxins is believed to involve at least 16 different enzymes. Some of the pathway genes encoding these enzymes, *nor-1*, *ver-1* and *omt-1*, and the regulatory gene, *afl-2/aflR(apa-2)*, have been cloned. We report here the organization and arrangement of the aflatoxin pathway genes on the chromosome. By determining overlapping regions of the inserts in cosmid and lambda clones, the four genes were located within a 60 kb DNA fragment in the order of *nor-1*, *afl-2/aflR*, *ver-1* and *omt-1*. This order is coincident with the order of the biosynthetic pathway with the regulatory gene, *afl-2/apa-2*, between *nor-1* and *ver-1* gene. Downsteam to the *omt-1* gene another two transcripts were identified. These transcripts are 1.4kb and 1.5 kb, respectively and may be involved in the aflatoxin biosynthetic pathway. Currently, the identities of the genes are being investigated. The relative order of and distances (in kilobase pairs) between these genes are shown below.



Note: 1, numbers indicating the length in kilobase;
2, arrows indicating the direction of transcription
3, assuming that each gene occupies 2 kb region

The arrangement of aflatoxin pathway genes in 60 kb DNA

A GUS Reporter Assay to Study Gene Expression and the Induction of Aflatoxin

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The research objective is to develop a method for identifying substances that induce or inhibit aflatoxin biosynthesis. Towards attaining this objective, the promoter regions of two aflatoxin biosynthetic pathway genes (*nor* and *ver*) from *Aspergillus flavus* were cloned into the GUS-reporter gene construct GAP4 to generate the promoter contructs, GAP12 and GAP13; respectively. These constructs were transformed into the *A. flavus* strain 656-2. Aflatoxin production and GUS activity were determined in the transformants after shifting the cultures from a medium that does not support aflatoxin production to a medium that does support its production. In transformants harboring the *ver* promoter-GUS construct GAP13, the GUS activity paralleled aflatoxin production in culture. Both aflatoxin and GUS activity were detected 18 hr after the culture medium shift. In the *nor* promoter-GUS (GAP12) transformants, aflatoxin accumulation was observed 6 hr earlier than in the GAP13 transformants. GUS activity was also increasing at this time point; however, the relative level of GUS activity was not proportionate to the aflatoxin concentration. Based on these data, the GAP13 transformants are good candidates for studying the induction of aflatoxin in situ and for indentifying substances that affect the expression of genes involved in aflatoxin biosynthesis.

Homology of the *omt*-1 gene from *Aspergillus parasiticus* with DNA of other fungal taxa.

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The *omt*-1 gene is involved in the formation of O-methyl-sterigmatocystin from sterigmatosystin, the penultimate step in the synthesis of aflatoxin B_1. This gene was isolated from *A. parasiticus* SRRC 143A. In this study, DNA of related fungal taxa were digested with the restriction enzyme *Eco*R1 and probed with the radiolabelled gene. DNA framents hybridizing to this gene were found in all *A. parasiticus* isolates tested, including one that had lost its ability to make aflatoxin and another that never produced aflatoxin, but did produce O-methyl-sterigmatocystin. There was also homology with *A. sojae*, a related koji mold that does not produce aflatoxin. *A. flavus* isolates including one aflatoxin-producer, one that had lost the ability to produce aflatoxin and one that had never produced aflatoxin all showed homology to the *omt*-1 gene.

Molecular characterization of *verA*, a gene necessary for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans*.

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Aflatoxin (AF) and sterigmatocystin (ST) are carcinogenic end products of the same biosynthetic pathway produced by many food contaminating *Aspergillus* spp., including *A. nidulans*. These mycotoxins are found in many food products and result in considerable health and economic costs to the US public. The degree of economic loss is aptly illustrated by the cash loss of the Texas corn crop in 1990 where it was estimated that 50% of the 400 million dollar crop had unacceptable levels of AF contamination. One approach to controlling AF/ST production is to identify and understand the molecular regulation of genes in the biosynthetic pathway. We have identified and characterized an *A. nidulans* gene, *verA*, needed to convert the AF precursor versicolorin A to ST. pJK2, a plasmid with *argB* inserted into an internal EcoRI site of the predicted *verA* coding region, was used to transform an *A. nidulans argB2*$^-$ mutant to *argB* prototrophy. Transformants exhibiting DNA patterns of a *verA* disruption event were assessed for ST production. They were found to accumulate versicolorin A while wildtype strains produced ST. This confirmed the prediction that *verA* encodes an enzyme necessary to convert versicolorin A to ST. These results demonstrate the ability to genetically engineer non-mycotoxin producing strains of *Aspergillus*. Such strains may be useful in a biocontrol strategy aimed at eliminating mycotoxin contamination in the field.

Introduction

Aflatoxin (AF) and its precursor, sterigmatocystin (ST), are carcinogenic polyketide mycotoxins produced by *Aspergillus parasiticus* and *Aspergillus flavus*. ST, but not AF, is also produced by *Aspergillus nidulans, Aspergillus ustus*, *Aspergillus versicolor* and other aspergilli. These fungi commonly infest many food products and both AF and ST may be found in oil seeds and their products, dairy products, meats, and other foodstuffs as well as livestock feed. Because they are secondary metabolites, they are not necessary for fungal growth and are only produced under specific conditions in field environments. Contamination of food products with these mycotoxins has led to documented animal deaths and they have been implicated as causal agents in human liver cancer.

The pathway intermediates leading to AFB_1 (the most toxic form of AF) have been characterized as: polyketide precursor > norsolorinic acid > averantin > averufanin > averufin > versiconal hemiacetal acetate > versicolorin A > sterigmatocystin > *o*-methyl sterigmatocystin > aflatoxin B_1. The AF/ST pathway - through ST - is found in the genetically well characterized *A. nidulans*. *A. nidulans* produces significant quantities of ST and offers many experimental advantages over *A. parasiticus* and *A. flavus* including: (1) both a sexual and asexual stage of development, unlike *A. parasiticus* and *A. flavus* which have only an asexual stage, (2) the availability of many developmental mutants of *A. nidulans* which may provide insight into secondary metabolite production as AF production has been related to developmental changes in strains of *A. flavus* and *A. parasiticus,* (3) the availability of primary metabolic mutants which may be used to better understand the relationship between primary and secondary metabolism, and (4) an extensive history as a model system to study fungal genetics and gene regulation, unlike *A. flavus* and *A. parasiticus*, which were not studied extensively until they were recognized as producers of AF. For these reasons, *A. nidulans* was successfully used as a model system to study penicillin production and is an ideal model for the study of AF/ST production.

Table I. High Performance Liquid Chromotography analysis of sterigmatocystin (ST) and versicolorin A (verA) levels in selected *Aspergillus nidulans* transformants grown on oatmeal.

Fungal Isolate [1]	µg verA[2]	µg ST
TJK1	55.7	924
TJK2	96.6	4.5
TJK6	77.3	BQL[3]
TJK8	65.9	5.5
TJK10	83.6	5.0
TJK4	79.8	4.0
TJK11	30.6	BQL
TJK7	79.1	2.0

1 transformants showing a *ver*A disruption event. TJK1 is a wild type control transformed with pSalArgB.

2 total µg of verA and ST produced by isolates grown on three gm of oatmeal for one week

3 BQL: below quantitation limit (0.5µg)

POSTERS

Crop Resistance

Postharvest resistance to aflatoxin contamination in maize inbreds previously tested in the field. R.L. Brown, T.E. Cleveland, K.W. Campbell, D.G. White, G.A. Payne and C.P.Woloshuk.

SUMMARY

Thirty-one inbreds, previously tested for resistance to aflatoxin contamination by Aspergillus flavus in a 1992 Illinois field trial, were tested for postharvest resistance using the resistance screening assay (RSA). Kernels from these inbreds and from the MAS:gk population were screened in three trials held at different times. MAS:gk has been shown previously, in field trials and by the RSA, to be highly resistant to aflatoxin contamination by A. flavus. Results indicate a high level of consistency between the three postharvest trials. This consistency was highlighted in the performance of entries 33-16 and NC232 as susceptibles and T115 and CI2 as resistant lines among those tested. The continuity of the resistance previously displayed by MAS:gk across both preharvest and postharvest studies was not evident in other lines tested. Overall, there was poor correlation between the postharvest tests and the Illinois field trial.

Entries T115, CI2 and M182, which displayed postharvest resistance in the screening experiments, and TEX6, which showed resistance in the field trial, but was among the most susceptible in the laboratory tests, were tested again to check for similarity with the resistance phenomenon displayed by MAS:gk. Nonwounded kernels and those pin-wounded through the pericarp to the endosperm, prior to inoculation with A. flavus, were assayed for aflatoxin contamination. MAS:gk, under the same experimental conditions, displayed resistance to aflatoxin B1 in both nonwounded and wounded kernels. Results indicate that M182 stands out among inbreds tested as being similar to MAS:gk in levels of toxin supported in nonwounded and wounded kernels.

To gain a better understanding of the fungal infection process in resistant and susceptible maize kernels, and to correlate fungal infection with toxin contamination, the GUS gene fusion system was employed in conjunction with the RSA. An A. flavus strain successfully transformed with the Escherichia coli B-D-glucuronidase gene in combination with the B-tubulin gene was inoculated onto kernels of inbreds T115, CI2, M182, TEX6, and two susceptibles, 33-16 and NC232. All kernels were pin-wounded as described above in the previous experiment. Results indicate that most fungal infection readily occurs in the embryo scutellum, despite the opportunity for entry presented by the wound into the endosperm. The fungus appears to travel through the aleurone layer towards the pedicel and embryo. Line M182, which in the previous experiment maintained resistance to toxin contamination after wounding, also showed very little fungal infection, even after 8 days incubation with A. flavus.

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Development of DNA delivery systems capable of introducing anti-fungal genes into peanut

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To develop efficient DNA delivery systems for the introduction of genes expressing anti-fungal characteristics into peanuts we are optimizing a number of genetic transformation protocols using a protoplast-based regeneration pathway. Efficient plant regeneration from protoplasts of a perennial wild peanut (*A. paraguariensis*) has been achieved by using a nurse culture method (1). Protoplasts isolated from cell suspensions were imbedded in agarose medium and co-cultured with nurse cells. Up to 20% of protoplasts formed microcalli within 3 weeks. Multiple shoots were produced from protoplast-derived callus colonies within 2 months after their transfer to plant regeneration medium. Modifications of this method have resulted in the successful regeneration of plants from immature cotyledon-derived protoplasts of cultivated peanut (2). Shoot bud formation has been observed in protoplast-derived calli from 7 peanut cultivars and breeding lines using modified plant regeneration conditions. Regenerated plants were normal in appearance, and fertile. Further efforts are being made to improve the frequency of plant regeneration.

Conditions for efficient PEG- and electroporation-mediated transformation are being defined by monitoring protoplast regeneration frequencies and transient GUS expression. A number of DNase inhibitors were tested for their effect on transformation efficiency. DNA plasmids containing different selection marker genes were introduced into peanut protoplasts for the evaluation of selection efficiency and their effects on plant regeneration. Plasmids containing virus coat protein (CP) genes were also constructed and introduced into peanut protoplasts as models for gene expression. Expression of CP genes has been detected in progeny of putative transgenic plants using ELISA. Confirmation of DNA integration is being performed.

A technique for rapid and efficient plant regeneration from hypocotyl tissues using thidiazuron (TDZ) has been developed. This technique provides an alternative explant for peanut transformation research using tissue electroporation, *Agrobacterium* and microprojectile bombardment approaches. Hypocotyl tissues from mature seeds were exposed to TDZ for one week and then cultured to hormone-free MS medium for 4-5 weeks. Over 200 shoots developed from a single explant via organogenesis. Normal plantlets were readily obtained by subculturing individual shoots to MS medium containing NAA. Regenerated plants were phenotypically normal and produced seeds in the greenhouse. Comparable regeneration frequencies were observed from all tested peanut genotypes, including 10 major US cultivars and two related *Arachis* species. Using this multiple shoot regeneration technique, hygromycin-resistant shoots were recovered after tissue electroporation with plasmids containing the *hpt* gene. When TDZ-treated tissues were inoculated with *Agrobacterium tumefaciens* containing a binary vector harboring kanamycin-resistance and virus CP genes, and following an extended period of selection, 37 out of 48 surviving plantlets tested positive for CP expression. Efforts are being made to confirm the identity of putative transformants by DNA analysis.

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Title: Progress Toward Generation of Transgenic Cotton Plants Resistant to A. flavus

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In this poster, we describe our progress in several areas that are pertinent to our ultimate goal of obtaining genetically engineered cotton that has increased resistance to fungal pathogens. The first area in which we focused our efforts was establishment of a system for the introduction of foreign genes into cotton that was relatively easy, reproducible, and quick. Our experiments during the past year, transforming cotton hypocotyl tissue with Agrobacterium cultures, and regenerating transgenic plants from callus tissue have not met these criteria.

We have recently focused most of our efforts on the utilization of a biolistic particle delivery system to introduce new genes into cotton. The biolistic approach does not depend on an Agrobacterium vector, and we are bombarding meristematic tissues that are easily and quickly regenerated. We have performed over 200 experiments with the BioRad Biolistic Helium Driven System. In these experiments we have optimized the system for maximum levels of expression of a marker gene (GUS) with minimum damage to the tissue. Tissues that have been bombarded using our optimized conditions form small plants within a four week period. The optimum conditions are pressures of 1100 or 1300 psi, distances of 6 to 9 cm from the particle containing disk, and a vacuum of 18 inches of mercury. These conditions were determined by testing 5 different particle delivery pressures, 4 different distances of the tissue from the delivery system, age of the tissue when bombarded, and two different particle sizes. After treatment with one set of conditions, tissues were histochemically stained for GUS activity, and scored based on the amount of stained tissue. Tissues were also scored based on the viability of the tissue following bombardment.

We have also optimized two different assay systems for the detection of the GUS marker gene. We plan to use this marker to trace the successful transformation of cotton plants. A colorimetric assay may be used for the detection of GUS activity. Using standard methods, we have prepared plant extracts from tobacco plants that are transformed with GUS to determine the sensitivity of the colorimetric assay for this enzyme. We are able to detect GUS activity in 0.1 microgram of crude protein extract. and 0.001 units of purified GUS. A second method for detection of GUS involved the use of a substrate that fluoresces when cleaved by GUS. This assay is more sensitive than the colorimetric assay, however cotton leaf extracts exhibit some intrinsic fluorescence. Our extraction procedure for fluorescence has been modified to include polyvinyl pyrrolidone and passage of the extracts over G-50 columns to eliminate the intrinsic fluorescence. Either of these assays is suitable for screening large numbers of cotton plants for the presence of the GUS marker.

Using the optimized GUS protocol, we have bombarded over 500 plant meristem explants. We are in the process of screening these plants for GUS activity. Our next studies will involve the biolistic bombardment of cotton tissues with genes that may confer increased resistance to fungal pathogens.

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